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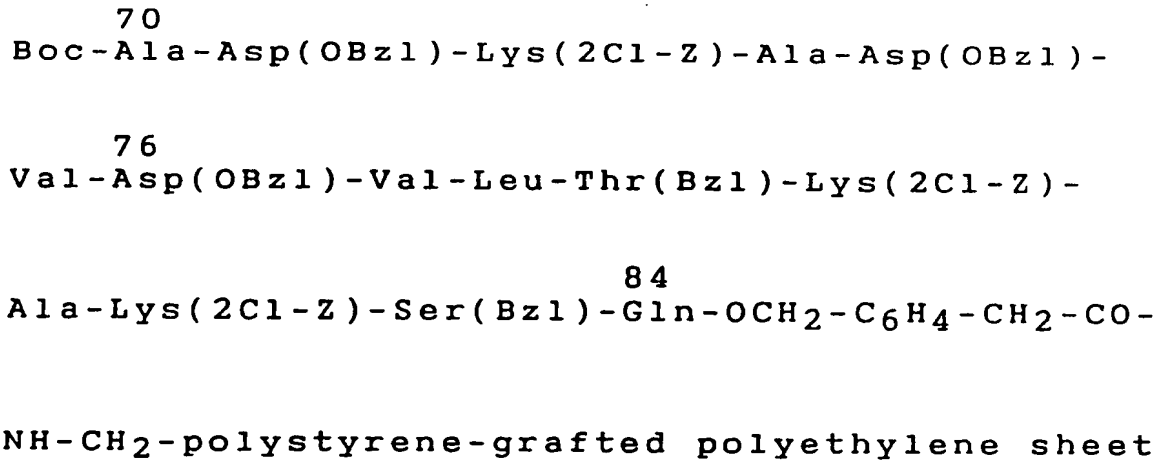

(57) Abstract

A solid-phase support suitable for use, for example, in the field of bioassays, such as ELISA-type assays, comprises a polymer substrate grafted with polystyrene chains which optionally further bear substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate being functionalized with a chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein upon reaction of the functionality with an amino acid, a peptide or a protein.

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SOLID SUPPORTS FOR USE IN SOLID-PHASE BIOSYSTEMS

FIELD OF THE INVENTION

The present invention concerns solid supports which are particularly well suited for use in solid-phase biosystems, notably bioassays, especially immunoassays, e.g. immunoassays employing an ELISA (Enzyme Linked Immunosorbent Assay) technique. In particular, the invention concerns a polymer substrate grafted with polystyrene chains as the solid support, the polystyrene chains optionally further bearing substantially non-reactive substituents and having an estimated molecular weight, not including optional substituents, of at least 200,000 and being functionalized with a chemical functionality upon which there may be established peptides and proteins, as well as amino acids, which are covalently bound in a substantially permanent fashion. The invention employs largely conventional chemical methodology, and the solid supports of the invention are suited for use with or without being attached to or positioned on a further material providing mechanical support, e.g. a mechanical supporting material which facilitates the fashioning of the solid supports of the invention in a particular form, such as, e.g., the form of a microtiter tray (also known as a microtiter plate).

BACKGROUND OF THE INVENTION

Present day solid-phase methods for performing bioassays, notably immunoassays of the type in which an antibody present in a fluid, such a body fluid, is detected or quantitated via its interaction with an antigen which is attached in some manner to the surface of a solid-phase support, or, alternatively, in which an antigen present in a fluid, such a body fluid, is detected or quantitated via its interaction with an antibody which is attached in some manner to the surface of a solid-phase support, generally exploit the fact that molecules such as peptides and proteins, e.g. numerous antigens and/or antibodies, adsorb, i.e. become attached in a non-covalent manner, to the surface of certain plastics or polymers, e.g. polystyrene, or other materials, such as glass, the nature of this

non-covalent attachment being, however, generally poorly characterized. However, since this attachment or binding is, indeed, non-covalent and of limited strength, there will in general be an equilibrium between the support-bound species, e.g. an antigen or antibody, and the "free" (solution) species. There is thus, in general, little control of the extent to which, e.g., an antigen or antibody adsorbed on the surface of the support material is lost during the various treatments, e.g. washing, to which the support is subjected in the course of the assay procedure. The severity of this problem will, of course, depend to a large extent on the rate at which equilibrium between adsorbed and free species, e.g. antigen or antibody, is established, and in certain cases it may be virtually impossible to perform a quantitative assay of acceptable accuracy and/or reproducibility. Loss of adsorbed species during treatment of the support with body fluids, aqueous reagents or washing media will, in general, be expected to be most pronounced for species of relatively low molecular weight and/or relatively high hydrophilicity, such as small, antigenic peptides.

In order to overcome the problems associated with the above-described loss of species bound by adsorption to the surface of the support it is clearly desirable to ensure the attachment of the species in question to the support in an essentially permanent manner, and in this respect covalent attachment is clearly desirable.

Some effort has been made previously to develop a support material suited for the purpose of performing bioassays of the above-outlined type, i.e. a support to which species such as antigens or antibodies may be covalently attached so as to avoid some of the above-described difficulties. An example of a commercially available product intended for use in immunoassays, e.g. using an ELISA technique, for the determination of antibodies is the microtiter plate marketed under the name CovaLink® and available from Nunc A/S, DK-4000, Roskilde, Denmark. The latter microtiter plate is constructed of clear polystyrene to which is covalently attached a so-called "linker" group. This linker, which is an amine derivative, has a chain terminating in an amine function which permits the attachment of peptides via a carboxyl function of the latter,

covalent bond formation between the linker and a carboxyl function on the peptide in question being achieved, e.g., by transforming the carboxyl function to its *N*-hydroxysuccinimido derivative by reaction with *N*-hydroxysuccinimide and then performing a condensation reaction between the latter peptide derivative and the amine function of the linker in the presence of a suitable coupling agent, such as a carbodiimide, so as to form an amide bond between the linker and the peptide. As long as the peptide to be attached to the support contains only one free carboxy group (and no other substituents which might alternatively react with the linker to form a covalent bond), all the attached individual molecules of the peptide in question will be attached in a uniform manner. However, if the peptide of interest contains two or more carboxy groups, or any other groups having potential reactivity towards the linker, the possibility of alternative modes of covalent attachment to the support will arise, and the above-mentioned uniformity of the mode of attachment will no longer be certain. In such a situation there is the risk that a peptide which is attached to the support, and which is known to function in the free state as an antigen towards an antibody which is to be detected or determined, may to a significant and possibly variable extent be oriented in such a manner that the epitope(s) necessary for satisfactory binding to the binding site(s) on the antibody are blocked by the binding of the peptide to the linker and/or rendered sterically inaccessible. In such cases the degree of interaction between the covalently bound peptide, with its various alternative binding modes, and the antibody may thus be reduced to such an extent, or be irreproducible to such an extent, that a satisfactorily sensitive and/or precise assay cannot be achieved.

Another line of approach to the provision of antigenic peptides or proteins which are covalently attached to a solid support is to synthesize the peptides *in situ* on a solid-phase synthesis substrate by means of established methods, and in the following section a brief account of such methods is given:

Present day methods for the solid-phase synthesis of peptides or proteins are largely based on the original methodology developed by Merrifield for the step-by-step construction of peptides from

individual amino acids. This methodology employs a functionalized cross-linked styrene/divinylbenzene copolymer, the cross-linked copolymer having been formed by the polymerization of styrene monomer to which has been added a few per cent (typically about 2%) of divinylbenzene. This copolymer is generally provided in the form of beads or particles, often with a dominant particle size of 20-80 μm. The functionalization originally preferred by Merrifield [see e.g. J. Am. Chem. Soc. *85*, 2149 (1963)] was a functionalization of the aromatic rings of the copolymer with chloromethyl groups, introduced via reaction of the solid copolymer with $SnCl_4$/chloromethyl methyl ether, although a number of other functionalities, including aminomethyl, α-aminobenzyl and α-amino-4-methylbenzyl, have subsequently been employed. Regardless of its nature, the purpose of the functionality is normally to form an anchoring linkage between the copolymer solid support and the C-terminal of the first amino acid which it is desired to couple to the solid support. More recent refinements of the Merrifield methodology have included the further introduction, between a functionality (e.g. one of the above-mentioned functionalities) on the polystyrene chains and the C-terminal of the first amino acid which is to be coupled, of a bifunctional linker (also called a "spacer" or "handle" groups) whose reactivity is tailored *inter alia* to meet desired requirements with respect both to the coupling of the first amino acid to the solid support and/or to the ease with which the completed, synthesized peptide or protein chain is cleaved from the solid support. Examples of such spacer groups include the phenylacetamidomethyl (Pam) and the *p*-alkoxybenzyl ester systems. A recent review dealing with the development of solid-phase peptide synthesis methodology since its introduction by Merrifield is given by Barany et al. [Int. J. Peptide Protein Res. *30*, 705-739 (1987)].

An example of the application of solid-phase peptide synthesis methodology for the provision of antigenic peptides which are covalently attached to a solid support and which as such can be employed in, e.g., an ELISA method is provided by the work of Geysen et al. [Proc. Natl. Acad. Sci. USA. *81*, 3998-4002 (1984) and *82*, 178-82 (1985)]; their method involves the use of acrylic acid-grafted polyethylene rods as the solid-phase synthesis substrate. However,

there are reports in the literature which indicate that the purity of the peptides synthesized by this method is often very poor, and this casts some doubt as to its reliability in connection with some (especially quantitative) immunoassays.

BRIEF DISCLOSURE OF THE INVENTION

The present invention provides solid supports which are well suited for use in solid-phase bioassays, in particular solid-phase immunoassays, and to which peptides or proteins, as well as amino acids, may be attached via a substantially permanent covalent bond and in a uniform manner, or upon which peptides or proteins may be built up by stepwise synthesis and then remain attached via a substantially permanent covalent bond. The invention also provides corresponding solid supports fashioned in a form which is well suited for a bioassay, e.g. the form of a microtiter tray, and to which peptides or proteins are attached via a substantially permanent covalent bond.

The invention also relates to the use of such substrates in a bioassay procedure, e.g. an immunoassay procedure, such as an ELISA-type procedure or a radioimmunoassay procedure, for the detection or quantitative determination of a biologically active species.

The solid supports of the invention are based on a polymer substrate to which are grafted long and substantially non-cross-linked polystyrene chains which (presumably owing, at least in part, to easy steric access thereto) when functionalized with suitable chemical functionalities serve as particularly efficient solid-phase carriers on which, in particular, peptides or proteins may be synthesized or attached and remain covalently bound in a stable, substantially permanent manner.

Preferred aspects of the invention are based on the use of non-cross-linked polyolefins, especially polyethylene, as the polymer substrate to which polystyrene chains are grafted. The insolubility of such polymer substrates in all organic solvents at ambient temperature may be exploited to reshape the grafted polymer, since

the latter is still a thermoplastic material and is soluble at elevated temperatures in certain organic solvents, such as xylenes in the case of polyethylene.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Protection scheme for the solid-phase assembly of [Asp^{76}]-hPTH fragment (70-84) on 443 wt % polystyrene-grafted polyethylene.

Fig. 2. Analytical HPLC chromatograms of (A) crude H-Lys-Ala-Lys-Ser-Gln-OH, (B) crude H-Val-Asp-Val-Leu-Thr-Lys-Ala-Lys-Ser-Gln-OH, and (C) crude H-Ala-Asp-Lys-Ala-Asp-Val-Asp-Val-Leu-Thr-Lys-Ala-Lys-Ser-Gln-OH on μBONDAPAK™ C_{18} (300 x 3.9 mm, 10 μm). Buffer A: H_2O/0.095% CF_3COOH; buffer B: 90% acetonitrile/10% H_2O/0.072% CF_3COOH; flow rate 1.3 ml/min.

Fig. 3. The amino acid sequences of melittin-(7-21) and melittin-(7-21) analogs.

Fig. 4. Analytical HPLC chromatograms of crude melittin-(7-21) and analogs after low/high HF-cleavage (before lyophilization). Chromatogram 1 is that for crude melittin-(7-21), i.e. peptide 1, chromatogram 2 is that for crude peptide 2, etc. Buffer A: 5% CH_3CN/95% H_2O/0.0445% TFA; buffer B: 60% CH_3CN/40% H_2O/0.0390% TFA; linear gradient: 5-95% of B in 30 min.; flow rate 1.5 ml/min.; column: Vydac C_{18} (0.46 x 25 cm).

DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention provides a polymer substrate grafted with polystyrene chains, said polystyrene chains optionally further bearing substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate being functionalized with a chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety

and an amino acid, a peptide or a protein upon reaction of said functionality with an amino acid, a peptide or a protein.

By the term "substantially permanent covalent anchoring linkage" is meant a covalent bond which under the conditions prevailing in the course of the various reactions and treatments to which the amino acid-, peptide- or protein-bearing substrate is exposed (e.g. further coupling of protected and/or derivatized amino acids or peptides, removal of protecting groups, washing, exposure to body fluids, etc) during the various steps involved in applying it to a bioassay procedure is stable with respect to release or loss of the attached (coupled) species.

The term polymer substrate as used in the present context denotes any suitable polymer which may be grafted as described and which in itself is substantially insoluble in and inert towards the reagents and media used in the reactions and treatments of interest. Suitable polymers may be selected, for example, from polyamides, such as nylon, polyimides, poly(paraxylylenes), poly(halofluoroalkenes), such as poly(tetrafluoroethylene) or poly(chlorotrifluoroethylene), phenol-formaldehyde polymers and polyolefins, such as polypropylene and polyethylene. The polymer substrate may be fashioned in any suitable form, for example a sheet, film, bead, pellet, disc, ring, tube, rod or net. A particularly interesting form is that of an "immuno-stick" (*vide infra*) or functional equivalent thereof. In preferred embodiments of the invention, the polymer substrate is polyethylene, e.g. low-density polyethylene in the form of a sheet or film or, for certain purposes, a net, although experiments show (*vide infra*) that high-density polyethylene is also suitable, e.g. in the case of a substrate fashioned in the form of an immuno-stick. Another interesting form of, in particular, polyethylene which is of relevance in relation to the possibility of fashioning a polymer substrate which is to be supported on a mechanical support material (*vide infra*) into some desired form, e.g. the form of a microtitre plate, is shrink-film, i.e. film which, e.g., upon heating or warming shrinks to fit closely to the contours of an underlying surface.

The polystyrene chains grafted to the polymer substrate may be chains of polystyrene itself or of polystyrene which is substituted to some extent with non-reactive substituents, i.e. substituents which are not capable of reaction under the conditions prevailing in the subsequent chemical reactions or other treatments to which the substrate of the invention is exposed. Such substituents may suitably be, for example, alkyl substituents, such as methyl, ethyl, propyl or butyl, alkoxy substituents, such as methoxy, ethoxy, propoxy and butoxy, or aryloxy substituents, such as phenoxy. The substitution will preferably take the form of substitution in the aromatic rings by one or more substituents, e.g. one or more of the above-mentioned substituents, although substitution at non-aromatic carbon atoms of vinyl group origin may also be envisaged. In preferred embodiments of the present invention the polymer substrate is polyethylene, and the grafted polystyrene chains are chains of non-substituted polystyrene.

It is believed to be advantageous, particularly when a functionalized polystyrene-grafted polymer substrate according to the invention is to be used for a coupling reaction with an amino acid, a peptide or a protein which takes place in a non-aqueous medium, that the polystyrene chains grafted to the polymer substrate are of a molecular weight, not including optional substituents present on the polystyrene chains, of at least 200,000. In a further aspect of the present invention, polystyrene chains fulfilling this condition may suitably be formed by a substantially radical-initiated reaction between the polymer substrate and optionally substituted styrene monomer present in a solution of the monomer in an organic solvent. Experiments have shown (*vide infra*) that when a polyethylene sheet or film is grafted with polystyrene chains under conditions where the polyethylene sheet or film is immersed in solutions, of varying concentration, of styrene monomer in a solvent such as methanol, the radical-initiated reaction being achieved by γ-radiation, not only does a grafting reaction occur, but non-grafted (i.e. free) polystyrene chains are formed. While there at present is no obvious, straightforward way of determining accurately the molecular weight of the grafted polystyrene chains themselves, the molecular weight of the non-grafted polystyrene chains formed may readily be determined

by, e.g., so-called "size-exclusion chromatography". It has been found that when pure styrene monomer is used, i.e. no methanol is present, the molecular weight of the non-grafted polystyrene chains (denoted hereafter as "homopolymer") occluded in the sheet (and extracted from the sheet with dichloromethane) which are formed under a certain set of γ-radiation conditions is predominantly about 180,000, and that the predominant molecular weight of the homopolymer increases with increasing methanol content of the styrene monomer/methanol solution; for example, in 70:30 (v/v) methanol/styrene the predominant molecular weight (M_{peak}) is about 1,000,000.

Results obtained with polystyrene-grafted polyethylene sheet grafted to various extents give strong indications that the molecular weight of the homopolymer occluded in the sheet and that of the grafted polystyrene chains correspond quite well, as will be explained at greater length in the following:

Size-exclusion chromatography establishes a relationship between species molecular weight and retention volume, the so-called "calibration curve". The molecular weight of a given fraction of, for example, polystyrene homopolymer with a particular retention volume is determined by comparison with retention volumes for polystyrene standards of known molecular weight. However, since no polystyrene-grafted polyethylene standards of known chain molecular weight are available, the best that can be done is to compare with the retention volumes of polystyrene standards under the same solution conditions.

The grafted sheet, the homopolymer and the polystyrene standards may be dissolved, e.g., in hot xylene, and in several such experiments the molecular weight of the most abundant fraction (M_{peak}) of homopolymer was found to be ca. 1,000,000. The M_{peak} value found for the polystyrene-grafted polyethylene sheet on the basis of the above-mentioned comparison with retention volumes of polystyrene standards was ca. 3,000,000 and upwards [it can be envisaged that a certain proportion of the individual polyethylene chains may be grafted with more than one polystyrene moiety, and the M_{peak} valu. determined in the above manner for the polystyrene-grafted polyethylene may

consequently be similar to or higher than that for the corresponding occluded polystyrene homopolymer].

Further evidence for the validity of the above-described molecular weight estimation procedure can also be derived from the above-mentioned experiments as follows:

The abundance, n_i, of a particular fraction, i, of molecular weight M_i is proportional to the height of the distribution curve at the retention volume corresponding to M_i. The so-called "weight average molecular weight", M_w, is then given by:

$$M_w = \Sigma\, n_i \times M_i^2 \,/\, \Sigma\, n_i \times M_i,$$

while the so-called "number average molecular weight", M_n, is given by:

$$M_n = \Sigma\, n_i \times M_i \,/\, \Sigma\, n_i$$

Current theory concerning radical-initiated polymerisation predicts a M_w/M_n ratio (the "polydispersity") of 2.0. A value of ca. 2 was found for the homopolymer, and the value found for the polystyrene-grafted polyethylene was also ca. 2, which may be taken as an indication that the polystyrene chains grafted to the polyethylene substrate have grown in essentially the same manner as the homopolymer, thereby lending further credence to the molecular weight estimation procedure outlined above. The estimated molecular weights referred to in the present description and claims were estimated in the above-described manner.

It is thus believed that the molecular weight of occluded homopolymer formed under a given set of conditions (solvent, styrene concentration, temperature, γ-radiation intensity and duration of γ-irradiation) closely reflects the molecular weight of the grafted polystyrene chains formed under the same set of conditions, the molecular weight determined for the homopolymer thus being taken as an estimate of the molecular weight of the grafted polystyrene chains.

It is also believed that the density of grafting sites on the surface of a polymer substrate, i.e. the number of points of attachment of polystyrene chains per unit surface area, as well as the extent of cross-linking of the grafted polystyrene chains, is strongly influenced by the conditions under which grafting takes place, in particular by the nature of an organic solvent used in the grafting process. Hydroxylic organic solvents, particularly alcohols such as methanol, are relatively hydrophilic and are therefore anticipated to be among the poorer solvents which may be chosen to dissolve a relatively hydrophobic substance such as styrene monomer. Thus, the degree of solvation of the monomer by such a solvent is expected to be relatively low by comparison with the degree of solvation which would be expected with a more hydrophobic organic solvent, for example a halogenated aliphatic hydrocarbon such as dichloromethane (dichloromethane being a preferred reaction solvent in solid-phase peptide synthesis methodology, both in general and in the context of the present invention). It is believed that poor swelling or solvation of the grafted polystyrene chains during the grafting process in a solvent such as methanol maintains the mobility of the growing polystyrene chains at a low level thereby leading to retardation of the diffusion-controlled chain-termination processes and thus facilitating the growth of particularly long polystyrene chains.

An attractive feature of the high molecular weight of the grafted polystyrene chains in the context of the present invention is that when functionalized, they may be presumed to behave, with regard to their reactivity towards reagents dissolved in hydrophobic solvents, such as dichloromethane (which dissolves free polystyrene), to a large extent as though they were non-grafted (i.e. free) functionalized polystyrene chains in homogeneous solution; the ease with which functionalized, grafted polystyrene chains formed in accordance with the present invention can react with such dissolved reagents, including protected and optionally derivatized amino acids or peptides, may therefore be regarded as optimal. The apparent substantial absence of cross-linking between the polystyrene chains grafted to the polyolefin facilitates extensive swelling or solvation of the chains by a chlorohydrocarbon solvent, e.g. dichloromethane,

such as is generally preferred in solid-phase methods for the stepwise synthesis of peptides

As mentioned previously, in conventional solid-phase peptide synthesis procedures employing "Merrifield-type" methodology, the solid support used is normally a functionalized cross-linked styrene/divinylbenzene copolymer, the cross-linked copolymer having been formed by the polymerization of styrene monomer to which has been added a few per cent (typically ca. 2 %) of divinylbenzene. This cross-linking reduces the degree of swelling or solvation of the functionalized copolymer matrix relative to that prevailing for functionalized, grafted polystyrene chains formed in accordance with the present invention, and thereby correspondingly reduces the reactivity of the former matrix.

According to the invention, it is preferred that the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, is in the range of 300,000-1,600,000, in particular 400,000-1,400,000, preferably 600,000-1,200,000. The presently preferred estimated molecular weight of substantially all of the polystyrene chains is 700,000-1,000,000. It is believed that the higher estimated molecular weights of 400,000 and above are particularly advantageous, but on the other hand, the grafting of polystyrene chains of the very highest estimated molecular weights of about 1,000,000 and above appears to have a detrimental effect on the mechanical properties of the polymer substrate, in particular when the substrate is, as is often preferred, in the form of a sheet or film.

The degree of polystyrene chain grafting of the polymer substrate, that is, the weight percentage of polystyrene relative to the polymer substrate, depends, of course, on the length of the polystyrene chains, the grafting site density and the dimensions of the polymer substrate, and may vary within wide limits. Thus, in the case of, e.g., a sheet or film of polymer substrate of thickness in the range of 25 to 100 μm, the degree of polystyrene chain grafting may be, e.g., from about 5 to about 800% by weight, such as from about 10% to about 700%. Intermediate degrees of grafting, e.g. of the order of

100-300%, are believed to be particularly suitable in the context of preferred embodiments of the present invention, but both lower and higher degrees of polystyrene chain grafting may be of value in certain circumstances.

Thus, for the purposes of performing assays of the type outlined earlier (*vide supra*), where it is, for example, desired to be able to attach or synthesize peptides or proteins (e.g. antigens, antibodies, enzymes or hormones) on a functionalized substrate according to the invention, the provision, for example, of a polymer substrate with an intermediate degree of polystyrene chain grafting, such as, e.g., 100-300%, often 200-300% by weight(for a sheet or film of polymer substrate of thickness in the range of 25 to 100 μm) will often be appropriate; in some cases, for example when working with covalently attached species of high antigenicity, it may also be desirable to limit the loading capacity of the functionalized, polystyrene-grafted substrate, and this can suitably be achieved by regulating the extent of functionalization of the substrate. There may, however, be certain assay applications for which a degree of polystyrene chain grafting of, e.g, 200-600%, such as 400-600%, may be more suited.

From an overall point of view, the practical upper limit of the degree of grafting for a sheet or film of polymer substrate of thickness in the range of 25 to 100 μm (as employed according to preferred embodiments) will often be about 500-600% by weight. On the other hand, the lowest degrees of grafting which are practicable will normally not be below about 40% for such a thin sheet or film. From a compromise point of view with regard to achieving satisfactory yield and efficiency of peptide or protein syntheses performed on the functionalized, grafted sheet, while at the same time retaining suitable mechanical strength of the grafted sheet or film, an upper practical range of degree of grafting seems to be about 200-400% by weight.

As will be apparent from a number of the examples provided to illustrate the invention, it is possible to functionalize the polystyrene-grafted polymer substrates as employed in the invention with other functionalities which instead of ensuring substantially

permanent covalent attachment of a peptide or protein make it possible to achieve facile release of, e.g., a peptide synthesized on the substrate in question. It is apparent from these examples that the yield of the synthesized peptides can be very high and that the "crude" (i.e. unpurified) peptides are of very high purity, and these examples are included simply to demonstrate and substantiate the fact that the general methodology employed in the context of the present invention for the stepwise building-up of peptides or proteins on the functionalized, polystyrene-grafted polymer substrate leads to attached species in high purity and in good yield. The essential and important conceptual distinction between the present invention and the purely synthetic approach described above is the provision, according to the present invention, of an anchoring functionality which ensures the continued, covalent attachment of the peptide or protein bound to the functionalized substrate; the chemistry involved in the actual *building-up* of a peptide or protein on the functionalized substrate is, however, essentially the same in the two cases, and for this reason one may confidently expect the peptides or proteins assembled on, and remaining attached to, a functionalized substrate of the present invention to be of high quality (uniformity) and thus, as explained previously, very well suited for application to biosystems such as bioassays.

Some of the results referred to above concerning the synthesis and isolation of peptides have been published (Berg et al., J. Am. Chem. Soc. *111* (1989) 8024). However, the latter publication neither mentions nor in any way implies the possibility of exploiting either functionalized or peptide-bearing substrates of the type in question for any form of bioassay procedure.

It should be mentioned that isolated peptides or proteins prepared by the above-mentioned method are ideally suited to attachment, if so desired, to a functionalized substrate according to the present invention.

As mentioned above, in one aspect of the invention, the polystyrene-grafted polymer substrate is formed by a substantially radical-initiated reaction between the polymer substrate and optionally substitu-

ted styrene monomer present in a solution of said monomer in an organic solvent. As also mentioned above, it is advantageous, from the point of view of obtaining long, substantially non-cross-linked polystyrene chains, to perform the grafting in a solvent in which the growing polystyrene chains are poorly swelled or solvated, such as a hydroxylic organic solvent, in particular an alcohol. Preferred alcohols for this purpose are C_{1-4} aliphatic alcohols. In practice, methanol has been found to be a most suitable solvent, but it is contemplated that also, e.g., ethanol, propyl and isopropyl alcohols, and n-butyl, iso-butyl, sec-butyl and tert-butyl alcohols will be applicable.

The volume percentage (% v/v) of optionally substituted styrene in the solution used for the grafting, such as a solution in a solvent which swells or solvates the growing polystyrene chains poorly, e.g. a hydroxylic solvent as explained above, in particular an alcohol as explained above, such as, e.g, methanol, has a marked influence on the molecular weight of the grafted polystyrene chains formed, in that, at least up to a certain point, the chain-length-increasing effect of the solvent is greater, the greater the volume percentage of the solvent in the solution. Thus, while the volume percentage of optionally substituted styrene in the solution may be within a very broad range, such as between 1 and 95%, this volume percentage will normally be in the range of 10 to 90%, more usually 20 to 80%. A very interesting range for the volume percentage of the optionally substituted styrene in the solution is between 25 and 50%, and as will appear from the examples, a range of 25 to 35%, in other words about 30% by volume, has been found in practice to give grafted substrates with excellent properties. An indication of the relation between the volume percentage of styrene in methanol during the grafting process and the resulting estimated polystyrene chain lengths appears from the below-mentioned experiments on the relationship between the volume percentage of the optionally substituted styrene in methanol and the molecular weight of the generated homopolymer at a constant γ-radiation dose and dose-rate.

The grafting process is very suitably performed by γ-irradiation in the absence of oxygen and at substantially ambient temperature or

slightly elevated temperature, the pressure being equal to the total vapour pressure of the liquid components, optionally supplemented by a moderate pressure of an inert gas such as argon, the total pressure then amounting to about 1 atmosphere. Suitable ways to remove oxygen from the reaction system are to subject the system to repeated freeze-thaw cycles on a high vacuum apparatus, or to subject the system to sonication followed by flushing with argon. The γ-irradiation is suitably performed at a dose rate in the range of from about 1 to about 100,000 Gy/hour, in particular about 200-5000 Gy/hour, such as about 300-1000 Gy/hour. It is believed that the intensity of the irradiation is of considerable importance to the obtainment of the desirable configuration with long, substantially non-cross-linked polystyrene chains; if the intensity is too high, the free radical formation will be so high that the grafting will tend to involve a greater number of shorter chains and perhaps a higher degree of cross-linking, both of which are normally not desired.

On the whole, the optimization of chain length and grafting (as well as the optical properties of the support, which in the context of the present invention may be of relevance, notably when the support is a sheet or film) is performed via the choice of polymer, optionally substituted styrene monomer, reaction mixture, radiation dose-rate, and temperature during irradiation.

While the above-described method involving γ-irradiation is the presently preferred method, it is contemplated that, e.g., polystyrene-grafted films may suitably be prepared using a different strategy involving conventional radical initiators, such as peroxides, for example hydrogen peroxide, benzoyl peroxide or diacetyl peroxide, or azo compounds as the radical-forming principles. Other radical-forming principles which may be employed are, e.g., ozone and UV-radiation; another particularly interesting radical-forming principle is an electron beam. The important thing is that the method used for the radical generation be one which is suitable for relatively well-controlled radical-initiated growth of the polystyrene chains. It is believed that the conditions mentioned above concerning the importance of the properties of the solvent used

also apply in connection with these free radical initiation principles.

It is also contemplated that it may be possible to produce, for example, polystyrene/polyethylene block copolymers useful for the present invention in a manner which does not make use of radical initiation. Thus, for example, it is possible using anionic polymerization to synthesize a block copolymer of butadiene and styrene, in which the chain length of the two blocks can be precisely controlled. It is possible to hydrogenate this polymer in such a manner that the polybutadiene block is converted into polyethylene. The polyethylene formed will have such a regular structure that it, in the solid state, will form high-density polyethylene. It is critical to this method that the polyethylene part of the copolymer should form a coherent film. It is contemplated that this can be obtained in the following manner: the ethylene/styrene block copolymer is dissolved in a solvent in which the polystyrene part is soluble at room temperature and higher temperatures but in which the polyethylene part is only soluble when the solvent is hot. An example of such a solvent is xylene. The polymer solution is placed in a mould and slowly cooled to below the temperature at which polyethylene precipitates. When the polyethylene film has been formed, the rest of the solvent is removed.

It is further contemplated that the latter-outlined alternative method of preparation may be extended to the preparation of other polystyrene/polyolefin block copolymers, for example polystyrene/-polypropylene block copolymer, by employing diene monomers other than butadiene, e.g. 2-methyl-1,3-pentadiene in the case of polystyrene/-polypropylene block copolymer.

While the polystyrene-grafted polymer substrate employed in the invention may be in any suitable form, such as explained above, very interesting embodiments are such in which it takes the form of a sheet or film. The thickness of the polymer substrate itself, for example a polyethylene substrate, which is the starting material for such a sheet or film, may vary within a wide range and will normally be from 10 to 10,000 μm, for most purposes preferably in the range 25

to 1000 μm, and typically in the range 25 to 100 μm such as 25 to 75 μm. The grafting process leads, of course, to an increase in the thickness. Thus, the thinner a sheet or film, the greater will the percentage increase in thickness be for a given set of grafting conditions. As an example, a thin grafted sheet or film may have a thickness in the range of 25 to 200 μm. For most purposes the thickness of a functionalized, polystyrene-grafted polymer substrate is suitably from 10 to 10,000 μm, preferably from 25 to 1000 μm, more preferably from 25 to 200 μm.

As already briefly mentioned, the grafted polymer, particularly in the form of a sheet or film, is a thermoplastic material and is soluble at elevated temperatures; reshaping after the grafting process is complete is thus contemplated as a possibility. Thus, in the case of a polystyrene-grafted polyethylene sheet or film, it is possible to dissolve the sheet or film in a suitable solvent and allow the solution to cool and the solvent to evaporate to obtain a new "casting" of the polymer support, e.g. as a thinner sheet or film, with the grafted polystyrene chains. The suitable solvent is one which, at a suitably high temperature, dissolves the polymer support with its grafted polystyrene chains (but with retention of the grafting) and which on cooling to a lower temperature is no longer capable of retaining the polymer substrate in solution, but still effectively swells or solvates the polystyrene chains. An example of such a solvent, useful, e.g., for polystyrene-grafted polyethylene, is a xylene or a mixture of xylenes. Preliminary experiments indicate (*vide infra*) that it is possible in this manner to coat certain types of support material, e.g. glass or poly(methylpentene) ("TPX"), with a thin film of polystyrene-grafted polyethylene, and it may be possible to functionalize supported films produced in this way so as to render them useful in the context of the invention.

A functionalized substrate in the form of a sheet or film has a number of advantages in the practical performance of the synthesis or attachment of a peptide or protein. Thus, e.g., sheet or film may easily be cut out in suitable sizes for arranging in the reaction vessels used, such as any type of known solid-phase peptide

synthesis reaction vessels, including flasks, beakers, microbeakers, funnels, wells, columns or nets.

In a further aspect of the invention, the amino acid, peptide or protein which is reacted with the functionality that facilitates the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein is (i) protected at the N-terminal and/or (ii) derivatized at the carboxyl terminal and/or, where relevant, (iii) side-chain protected. Thus, it is possible, for example, to attach in a specific and uniform manner molecules of a peptide which has a number of side-chains (e.g. several carboxy groups) at least some of which, if they were not in protected form, would be able to bind to the functionality on the substrate, thus leading (as explained earlier, above) to the possibility of alternative binding modes.

For the latter reason, functionalized, polystyrene-grafted polymer substrates according to the invention can provide an important advantage relative to, for example, the above-mentioned product registered under the name CovaLink®, as is explained in the following: Firstly, the reaction conditions necessary for the removal of side-chain protecting groups from peptides generally comprise the use of aggressive media containing HF or trifluoroacetic acid. Experiments carried out by the present inventors have shown that the "linker" employed in CovaLink® microtiter plates is ruptured or decomposed by these aggressive media, and it is thus clear that the covalent binding attaching a species to this substrate would thereby be completely abolished upon treatment with such media, with attendant loss of the species in question. Secondly, the attachment of, e.g., highly protected peptides to a solid-phase substrate requires the use of non-aqueous solvents, such as dichloromethane, which dissolve the polystyrene material of the CovaLink® plate, leading, *inter alia*, to loss of the linker groups. For the above-mentioned reasons it is then clear that it is not possible using CovaLink® plates to ensure the uniform attachment of, e.g., molecules of a peptide having side-chains which can compete (with each other and/or with the terminal carboxy group of the peptide) with respect to binding to the amine function of the linker.

As is clearly demonstrated in the present specification, the functionalized, polystyrene-grafted polymer substrates of the present invention are not subject to the above-described drawbacks.

In further aspects of the invention, a peptide or protein which reacts with the functionality on, or is attached via a substantially permanent covalent anchoring linkage to, a substrate according the invention is a peptide or protein selected from the group consisting of:

peptide and protein antigens which participate in an *in vivo* and/or *in vitro* antigen/antibody reaction with an antibody,

antibodies which participate in an *in vivo* and/or *in vitro* antibody/antigen reaction with an antigen,

peptide and protein hormones, and

enzymes.

A non-limiting list of some examples of peptide hormones which may be of relevance in the context of the invention are: vasopressin, growth hormone releasing factor, substance P, bradykinin, parathyroid hormone, calcitonin, gastrin, growth hormone, oxytocin, neurotensin and secretin.

With regard to the coupling or immobilization of enzymes to a substrate of the invention, it is believed that the relatively hydrophobic nature of the polystyrene chains themselves favours the attachment, in particular, of relatively hydrophobic enzymes, e.g. lipases. By functionalizing the substrate with an amino-terminated functionality, such as aminomethyl (*vide infra*), and using a coupling reagent such as glutaric dialdehyde or a carbodiimide, it is possible that the covalent attachment of such a hydrophobic species may be achieved relatively straightforwardly.

The chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between an amino acid, a peptide or a protein and the functionalized polystyrene moiety is suitably a member of, or is derived from a member of the group consisting of:

chloro-, bromo- and iodo-substituted alkyl,

amino-substituted alkyl,

hydroxy-substituted alkyl,

the functionality, if derived from any of said group, being a functionality with a spacer group.

According to suitable embodiments of the invention, chloro-substituted alkyl is chloromethyl, amino-substituted alkyl is aminomethyl, and hydroxy-substituted alkyl is hydroxymethyl. The use of other moieties such as, e.g., terminally chloro-, amino- or hydroxy-substituted C_2-C_6 straight-chain alkyl groups selected from ethyl, n-propyl, n-butyl, n-pentyl and n-hexyl, may be appropriate for certain applications, for example to take account of certain steric requirements of the species to be attached to or built up on the functionalized substrate.

Concerning the initial functionalization of the polystyrene-grafted polymer substrate, more than fifty functionalization methods methods have been described in connection with traditional solid-phase peptide synthesis (see Barany and Merrifield in *The Peptides*, Vol. 2, Academic Press, New York, 1979, pp. 1-284, and Stewart and Young, *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Company, Illinois, 1984), of which reactions for the introduction of chloromethyl (via a chloromethyl methyl ether / $SnCl_4$ reaction, aminomethyl (via a *N*-hydroxymethylphthalimide reaction; see Mitchell et al., *Tetrahedron Lett.*, 3795, (1976)) and benzhydrylamino (Pietta and Marshall, *J. Chem. Soc.*, 650 (1970)) groups are the most widely applied in approaches directed to the synthesis and isolation of peptides or proteins. Other reactive functionalities which have been

initially introduced include 4-methylbenzhydrylamino and 4-methoxybenzhydrylamino.

Not all of of these established methods are, however, useful within the context of the present invention, since the fundamental requirement in the context of the invention is that the functionality established on the substrate forms a substantially permanent covalent bond to the attached species. Preferred embodiments of the present invention employ aminomethyl, in that aminomethyl in itself functions very satisfactorily as functionality owing to the ease with which the amino group can be reacted to form amide bonds to an activated carboxylic acid group, e.g. of an amino acid, a peptide or a protein. This reactivity also makes the aminomethyl group particularly advantageous with respect to the incorporation of "linker", "spacer" or "handle" groups at one end of the spacer-forming reagent.

In established solid-phase techniques for the synthesis and isolation of peptides or proteins, a main purpose of the incorporation of a linker or spacer has generally been to facilitate the removal of the completed peptide or protein intact from the synthesis substrate. In this connection a vast number of relevant spacer- or handle-forming bifunctional reagents have been described (see Barany et al., *Int. J. Peptide Protein Res.*, *30*, 705-739 (1987), especially reagents which are reactive towards amino groups, such as the amino group in the aminomethyl function, including a 4-(haloalkyl)aryl-lower alkanoic acid such as 4-(bromomethyl)phenylacetic acid, a Boc-aminoacyl-4-(oxymethyl)aryl-lower alkanoic acid such as Boc-aminoacyl-4-(oxymethyl)phenylacetic acid and numerous others.

An alternative strategy concerning the introduction of spacer or handle groups is the so-called "pre-formed handle" strategy (see Tam et al., *Synthesis*, 955-57, (1979)), which offers complete control over coupling of the first amino acid, and excludes the possibility of complications arising from the presence of undesired functional groups not related to the peptide or protein synthesis. In this strategy, spacer or handle groups, in general spacer or handle groups of the same types as described above, are reacted with a first amino acid which it is desired to anchor to the solid support, the amino

acid being *N*-protected and optionally protected at other side-chains which are not relevant with respect to the building-up of the desired peptide or protein chain. Suitable *N*-protecting groups are Boc, normally in combination with benzyl groups for the protection of side chains, and Fmoc, normally in combination with *t*-butyl for the protection of any side chains (Boc = *t*-butyloxycarbonyl; Fmoc = 9-fluorenylmethyloxycarbonyl), although a number of other possibilities exist which are well known in conventional solid-phase peptide synthesis.

Thus, in those cases in which a spacer or handle group is desirable, a first amino acid to be coupled to the solid support can either be coupled to the free reactive end of a spacer group which has been bound to the initially introduced functionality, for example aminomethyl, or can be reacted with the spacer-forming reagent, which in turn is then reacted with the initially introduced functionality.

As already discussed, linkers or spacers used within the context of the present invention must be of a type which ensures that a covalently attached species remains attached to the substrate, and for this purpose the relevant linkers will generally be selected from species differing from those mentioned above in connection with purely synthetic aspects. Thus, in preferred aspects of the present invention the functionality is derived from an amino-substituted alkyl group, such as aminomethyl, and the functionality comprises a spacer group derived from the group consisting of straight- and branched-chain ω-aminoalkanoic acids, preferably a spacer group derived from the group consisting of 6-aminohexanoic acid, 5-aminopentanoic acid, 4-aminobutanoic acid and 3-aminopropanoic acid.

Another interesting functionality may be one which is derived from a halo-substituted, e.g. chloro-substituted, alkyl group, such as chloromethyl, and comprising a spacer group derived from a mercaptoamine, e.g. 2-mercaptoethylamine (2-aminoethanethiol). Such mercapto compounds may be reacted, e.g. after deprotonation of the thiol group with strong base, with, e.g., a chloromethyl group to give a stable, amino-terminated linker group attached to the

polystyrene chains of the substrate via a $-CH_2-S-$ bond. An alternative way of producing this same linker would be to allow an initially introduced chloromethyl group to react with HS^- ion, e.g. in the form of NaHS, to give a thiomethyl group, and then react the latter with, e.g., 2-chloroethylamine via a base-induced substitution reaction.

The linkers mentioned above in the context of the invention all have a terminal amino group by means of which the C-terminal of an amino acid, a peptide or a protein can be attached covalently, and substantially permanently, via an amide bond; in preferred embodiments of the invention, the covalently bound species (e.g. an antigenic peptide) established on the substrate of the invention is attached in this manner. However, such a covalently attached species may, in principle equally well be attached to the substrate in the reverse manner, i.e. via the N-terminal of, e.g., an antigenic peptide. This will of course require that the functionality of the substrate is adapted to form a satisfactorily permanent covalent bond to the N-terminal of the species in question. This may be achieved in the context of the invention by, for example, employing a linker which terminates in a free carboxy group. One way of achieving this is to couple (for example by using a carbodiiide reagent) one carboxy function of a dicarboxylic acid moiety, such as succinic or glutaric acid, to an initially introduced aminoalkyl, e.g. aminomethyl, functionality on the substrate via formation of an amide bond.

In a further aspect of the present invention, a functionalized, polystyrene-grafted polymer substrate within the context of the invention is supported on a substantially chemically inert and optionally light-transparent support material, and suitable support materials may be selected from the group consisting of: glass, ceramics, poly(methylpentene) (for example "TPX"), polytetrafluoroethylene, polypropylene, polyethylene, hard PVC, and silicone rubbers. In a further aspect, such a supported substrate may be fashioned in the form of, for example, a microtiter plate, a test tube, a beaker, a flask, a tray, a Petri dish, a strip, a rod or a fibre. By the term "substantially chemically inert" is meant

substantially unaffected by any treatments to which the support material in question is exposed in the course of the attachment or affixing of the functionalized, polystyrene-grafted polymer substrate, or in the course of reagent treatments or washing of the latter functionalized substrate. Light transparency of a support material may be advantageous if it is desired, e.g., to monitor a colour-producing reaction taking place in the course of a bioassay procedure. An example of such a reaction is a colour reaction in an ELISA procedure, the colour arising typically from release, via a process (such as hydrolysis) catalyzed by an enzyme conjugated, for example, to an antibody which undergoes an antigen/antibody reaction with an antigenic peptide or protein attached to the functionalized substrate, of a species producing a colour in the presence of an added colorimetric reagent. Transparency of the support material may then facilitate spectrophotometric monitoring of the colour reaction in question by measurement of transmitted light intensity at a chosen wavelength or wavelengths; this may be be particularly convenient if the supported, functionalized substrate of the invention is in the form of, e.g., a microtiter tray with wells.

Yet another aspect of the invention provides a polymer substrate grafted with polystyrene chains and to which a peptide or protein is coupled, said polystyrene-grafted substrate preferably being fashioned in a form selected from the group consisting of a microtiter plate, a test tube, a beaker, a flask, a tray, a Petri dish, a strip, a rod and a fibre, and being supported on a substantially chemically inert and optionally light-transparent support material, said polystyrene chains optionally further bearing substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate bearing a substantially permanent covalent anchoring linkage via which said peptide or protein is coupled. The peptide or protein coupled to such a substrate may, for example, be

a peptide and protein antigen which participates in an *in vivo* and/or *in vitro* antigen/antibody reaction with an antibody,

an antibody which participates in an *in vivo* and/or *in vitro* antibody/antigen reaction with an antigen,

a peptide or protein hormones, or

an enzyme,

The support materials for such a substrate, and the thickness of the peptide- or protein-bearing polymer substrate part of such a supported substrate, are suitably as mentioned above for functionalized, polystyrene-grafted polymer substrates of the invention.

The invention also provides a method for the preparation of a polymer substrate grafted with polystyrene chains, said polystyrene chains optionally further bearing substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate being functionalized with a chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein upon reaction of said functionality with an amino acid, a peptide or a protein, the method comprising:

a) subjecting a polymer substrate immersed in a solution of optionally substituted styrene monomer in an organic solvent to a treatment leading to the formation of free radicals such that polystyrene chains which are grafted to the polymer substrate are formed,

b) washing the polystyrene-grafted polymer substrate produced according to step (a) with a suitable solvent or solvents so as to substantially completely remove non-grafted, optionally substituted polystyrene chains and optionally substituted styrene monomer, and

c) functionalizing at least part of said grafted polystyrene chains with a chemical functionality facilitating the formation of an substantially permanent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein.

In a further aspect, the invention also provides a method for the preparation of a polymer substrate grafted with polystyrene chains and supported on a substantially chemically inert and optionally light-transparent support material, said polystyrene chains optionally further bearing substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate being functionalized with a chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein upon reaction of said functionality with an amino acid, a peptide or a protein, the method comprising:

a) attaching or affixing a polymer substrate to said support material,

b) subjecting the supported polymer substrate immersed in a solution of optionally substituted styrene monomer in an organic solvent to a treatment leading to the formation of free radicals such that polystyrene chains which are grafted to the polymer substrate are formed,

c) washing the supported, polystyrene-grafted polymer substrate produced according to step (b) with a suitable solvent or solvents so as to substantially completely remove non-grafted, optionally substituted polystyrene chains and optionally substituted styrene monomer, and

d) functionalizing at least part of said grafted polystyrene chains with a chemical functionality facilitating the formation

of an substantially permanent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein.

In an alternative aspect, the invention also provides a method for the preparation of a polymer substrate grafted with polystyrene chains and supported on a substantially chemically inert and optionally light-transparent support material, said polystyrene chains optionally further bearing substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate being functionalized with a chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein upon reaction of said functionality with an amino acid, a peptide or a protein, the method comprising:

a) subjecting a polymer substrate immersed in a solution of optionally substituted styrene monomer in an organic solvent to a treatment leading to the formation of free radicals such that polystyrene chains which are grafted to the polymer substrate are formed,

b) washing the polystyrene-grafted polymer substrate produced according to step (a) with a suitable solvent or solvents so as to substantially completely remove non-grafted, optionally substituted polystyrene chains and optionally substituted styrene monomer,

c) attaching or affixing the polystyrene-grafted and washed polymer substrate to said support material, and

d) functionalizing at least part of said grafted polystyrene chains with a chemical functionality facilitating the formation of an substantially permanent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein.

In the above-mentioned methods of the invention, the washing of the polymer substrate after completion of the grafting step serves several purposes: (i) For purely hygienic or environmental health reasons it is desirable to eliminate, in particular, residues of styrene monomers. (ii) The presence of significant amounts of non-grafted polystyrene in the grafted substrate may lead to irreproducible behaviour of the substrate when subjected to functionalization. (iii) From the point of view of providing a satisfactory and marketable commercial product for use in the field of bioassays, the presence of appreciable quantities of residues of starting materials is clearly unacceptable.

The method employed for attaching or affixing the polymer substrate, before or after the grafting and washing has taken place, to a support material will, of course, depend on the nature of the polymer substrate employed, and on the nature and shape of the support. It is difficult to provide general guidelines. An interesting method of attachment is the use of polymer substrate in the form of shrink-film (*vide supra*) to produce a polymer substrate which closely fits the contours of the underlying surface, this being feasible in the case of, e.g., polyethylene as polymer substrate.

The invention also relates to functionalized, polystyrene-grafted polymer substrates prepared by the above-described methods.

A further aspect of the invention provides a method for the preparation of a polystyrene-grafted polymer substrate bearing a peptide or protein thereon and supported on a substantially chemically inert and optionally light-transparent support material, the method comprising the steps of:

A) providing a supported polymer substrate grafted with polystyrene chains, said polystyrene chains optionally further bearing substituents which are not reactive under the conditions necessary to effect any of steps (B)-(G) below, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains

of the polystyrene-grafted polymer substrate being functionalized with a chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety and an at least *N*-protected and optionally carboxyl terminal derivatized amino acid or peptide, the functionalized, polystyrene-grafted polymer substrate being supported on a substantially chemically inert and optionally light-transparent support material,

B) coupling an *N*-protected and optionally carboxyl terminal derivatized amino acid or peptide to the functionalized polystyrene moiety,

C) in those cases where an amino acid has been coupled in step (B), and optionally in those cases where a peptide has been coupled in step (B), removing the *N*-protecting group from an *N*-protected amino or substituted amino group of the coupled and *N*-protected amino acid or peptide, such that reaction of the amino or substituted amino group of the coupled amino acid or peptide with a carboxyl group or an activated carboxyl group of a further amino acid or peptide is facilitated,

D) in those cases where step (C) has been performed, reacting said amino or substituted amino group of the last-coupled amino acid or peptide with a carboxyl group or an activated carboxyl group of a further *N*-protected amino acid or peptide so as to form a peptide bond between the two moieties,

E) in those cases where step (D) has been performed, optionally removing the *N*-protecting group from an *N*-protected amino or substituted amino group of the last-coupled *N*-protected amino acid or peptide, such that reaction of the amino or substituted amino group of the latter amino acid or peptide with a carboxyl group or activated carboxyl group of a further *N*-protected amino acid or peptide is facilitated,

F) in those cases where step E) has been performed, repeating steps D) and E) a desired number of times,

G) removing protecting groups possibly remaining on the amino acid moieties of the coupled peptide or protein chain.

When using the method to synthesize a peptide or protein *in situ*, the method will proceed as follows: Following the coupling of the first amino acid or peptide which is to be coupled, the next stage of the synthesis is the systematic elaboration of the desired peptide or protein chain. This elaboration involves repeated deprotection/coupling cycles. The temporary protecting group, such as a Boc or Fmoc group as described above, on the last-coupled amino acid or peptide is quantitatively removed by a suitable treatment, for example by acidolysis, such as with trifluoroacetic acid, in the case of Boc, or by base treatment, such as with piperidine, in the case of Fmoc, so as to liberate the N-terminal amine function of the last-coupled amino acid.

The next desired *N*-protected amino acid or peptide is then coupled to the N-terminal of the last-coupled moiety. This coupling of the C-terminal of an amino acid or peptide with the N-terminal of the last-coupled moiety can be achieved in several ways, for example by providing the incoming amino acid or peptide in a form with the carboxyl group activated by any one of several methods, including the initial formation of an active ester derivative, or the initial formation of an anhydride. Alternatively, the carboxyl group of the incoming amino acid or peptide may be reacted directly with the N-terminal of the last-coupled moiety with the assistance of a condensation reagent, for example dicyclohexylcarbodiimide or derivatives thereof.

Following the completed assembly of the desired peptide or protein chain, including protecting groups, the final step will be deprotection of the amino acid moieties of the peptide or protein chain.

It is thus possible by the latter method to achieve either of the following: (i) a peptide or protein can be attached directly and

covalently to the substrate; (ii) a peptide or protein can be synthesized in a stepwise manner using amino acid units or peptide units in any desired sequence, e.g. amino acid + amino acid +.... or amino acid + peptide + ... or peptide + amino acid +...., etc.

As discussed in some detail earlier, above, the substrates of the invention have the great advantage that it is possible to attach peptides having full side-chain protection, thereby ensuring uniformity of mode of attachment. Thus, in a further aspect of the above method, a peptide having side chains in protected form is coupled in step (B), and steps (C)-(F) are not performed.

A further aspect of the invention provides a method for the preparation of a polystyrene-grafted polymer substrate bearing a peptide or protein thereon and supported on a substantially chemically inert and optionally light-transparent support material, the method comprising the steps of:

A) providing a supported polymer substrate grafted with polystyrene chains, said polystyrene chains optionally further bearing substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate being functionalized with a chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety and a peptide or protein in its native state, the functionalized, polystyrene-grafted polymer substrate being supported on a substantially chemically inert and optionally light-transparent support material, and

B) coupling said peptide or protein to the functionalized polystyrene moiety.

The latter method will particularly relevant in cases where it is desired to covalently attach, e.g., a native antigen or antibody present in an aqueous medium such as a body fluid.

The invention further relates to the use of a functionalized substrate or a peptide- or protein-bearing substrate according to the invention as a carrier in a bioassay procedure for the detection or quantitative determination of a biologically active species, the species which is covalently attached, or which becomes covalently attached, being a peptide or protein having an epitope or binding site that can bind to a complementary binding site or epitope on said biologically active species.

The biologically active species in question may, e.g., be an antibody, in which case the the attached peptide or protein will be an antigen which undergoes an antigen/antibody reaction with the antibody. It may alternatively be an antigen, in which case the attached species will be a protein antibody which undergoes an antigen/antibody reaction with said antigen.

In a further aspect, the bioassay in question is a bioassay, such as an immunoassay, which is performed using an ELISA technique, for example as outlined previously and making use of spectrophotometric colour intensity measurements.

The following examples illustrate the invention, the abbreviations used being as follows:

LIST OF ABBREVIATIONS

Boc:	*tert*-butyloxycarbonyl
ClZ:	2-chlorobenzyloxycarbonyl
DCC:	N,N'-dicyclohexylcarbodiimide
DCU:	N,N'-dicyclohexylurea
Dhbt:	3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl
Dhbt-OH:	3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
DIEA:	N,N-diisopropylethylamine
DMF:	N,N-dimethylformamide
FABMS:	Fast atom bombardment mass spectrometry
Fmoc:	9-fluorenylmethyloxycarbonyl.

HOBt:	1-hydroxybenzotriazole
HPLC:	high performance liquid chromatography
Pam:	phenylacetamidomethyl
PE:	polyethylene
Pfp:	pentafluorophenyl
PP:	polypropylene
PS:	polystyrene
SEC:	size-exclusion chromatography
SPPS:	solid phase peptide synthesis
TFA:	trifluoroacetic acid
TFMSA:	trifluoromethanesulfonic acid
THF:	tetrahydrofuran

The abbreviations used for the various amino acids are in accordance with the recommendations of the IUPAC-IUB Commission of Biochemical Nomenclature [J. Biol. Chem., *247*, 977-983 (1972)], and refer in all cases to L-configuration amino acids.

EXAMPLE 1

(a) *Procedure for preparation of polystyrene-grafted polyethylene sheets*.

Styrene (99% Aldrich) was passed through basic alumina; in some cases it was further distilled from sodium or from calcium hydride. 20 ml of a 30% (v/v) solution of purified styrene in methanol was placed in an ampoule together with a rectangular strip of low-density PE sheet which had been washed in n-hexane. The sheet used had a thickness of 54 μm. The solution was thoroughly degassed by repeated freeze-thaw cycles on a high vacuum line and the ampoule was then sealed under vacuum. The ampoule and contents were then irradiated in a cobalt gamma-irradiation facility. The irradiation was carried out in two stages, the ampoule being moved from one location in the irradiation source to another between the two stages to ensure as homogeneous a dose distribution as possible. The dose rate was approximately 400 Gy/hour. The highest dose rate used was 417 Gy/hour and the lowest 339 Gy/hour. After irradiation the sheet was extracted in a Soxhlet apparatus with dichloromethane and dried. Specific data are listed in

Table 1(a). It is noteworthy that although there is a clear correlation between the irradiation dose and the extent of the grafting, there is much scatter in the data. This is partly due to the so-called "after effect", the polymerization process continuing to some extent after the irradiation is stopped. As an example of this effect the ampoule containing the sheet irradiated with a total dose of 3.4 kGy to yield a 450 % grafted sheet was left outside the irradiation source for 10 hours between the two stages of irradiation. Furthermore, the ampoule was first opened 10 days after completion of irradiation. A similar procedure was used for the sheet irradiated with a total dose of 2.0 kGy to yield 230% grafting.

TABLE 1(a)

Grafting of PE sheets in methanol/styrene (70% v/v)

Mass of PE sheet (g)	Irradiation dose (kGy) (Irradiation time in hours)	Graft %*	Duration of extraction with CH_2Cl_2 (hours)
0.2288	5.6 (14.0)	547	30
0.290	4.0 (10.0)	443	96
0.3322	3.4 (9.0)	450	330
0.2742	3.0 (8.1)	220	240
0.3866	2.9 (8.0)	285	170
0.3400	2.7 (6.7)	231	90
0.2398	2.4 (6.0)	173	120
0.3399	2.0 (6.0)	230	185
0.3502	1.7 (4.8)	200	182
0.3710	1.7 (5.0)	180	260
0.3456	1.0 (3.0)	75	56
0.3385	1.0 (3.0)	55	114§
0.3831	0.98 (2.8)	80	119

* : Graft % = [(mass of final sheet) -(mass of polyethylene)] x 100/(mass of polyethylene)

§ : The top of the sheet was damaged during the closure of the ampoule.

Sheets with graft % 46, 129 and 331, respectively, have also been prepared.

(b) *Alternative procedure for preparation of polystyrene-grafted polyethylene sheets.*

Styrene (99% Aldrich) was passed through basic alumina. 20 ml of a 30% (v/v) solution of purified styrene in methanol was placed in an ampoule together with a rectangular strip of low-density PE film. The sheet used had a thickness of 54 μm. The solution was thoroughly degassed by sonication followed by flushing with argon, and the ampoule was then sealed. The ampoule and contents were then irradiated in a cobalt gamma-irradiation facility. The dose rate was approximately 400 Gy/hour. After irradiation, homopolymer, i.e. non-grafted polystyrene chains, was removed by washing in toluene at 50°C. The film was then washed with dichloromethane and dried. Specific data for the preparation of a series of polystyrene-grafted PE films in this manner are given in Table 1(b)

TABLE 1(b)

Grafting of PE film in methanol/styrene (70% v/v)

Mass of PE film (g)	Irradiation dose (kGy)	Graft %*
0.34	ca. 1.0	ca. 50
0.38	ca. 1.1	ca. 100
0.35	ca. 1.9	ca. 200
0.39	ca. 3.1	ca. 300
0.29	ca. 3.9	ca. 400

(c) *Grafting procedure for preparation of polystyrene-grafted polyethylene "immuno-sticks".*

In the case of a particularly interesting form of polyethylene substrate, namely so-called "immuno-sticks" (*vide infra*, Example 12), the grafting procedure was carried out in a gamma-irradiation

facility differing from that used in connection with the other examples given herein. This alternative gamma-irradiation facility has a considerably larger capacity (i.e. can accommodate a larger number or larger volume of objects to be irradiated) than that otherwise used, and delivers gamma radiation at a mean dose rate (ca. 1.2 kGy/hour) which is ca. 3 times higher (and at an irradiation temperature which is ca. 0.5 °C higher) than that (ca. 400 Gy/hour) in the facility otherwise used. As a rough guide, and as a consequence of incomplete radiation absorption, the total dose employed in this alternative facility of higher dose rate in order to attain a given percentage of grafting should be ca. 1.7 (i.e. $\sqrt{3}$) times higher than that employed in the other facility.

Irradiation of substrates to be grafted with PS was normally carried out for 4 hours with the substrate(s) in question immersed in methanol/styrene (70% v/v) contained in a rectangular aluminium vessel (400 X 300 X 50 mm) provided with a tightly fitting lid. Upon completion of the irradiation the solvent mixture was removed form the vessel, which was then filled with toluene, closed and left to stand overnight. The irradiated (and thereby PS-grafted) and toluene-washed substrate(s) was/were air-dried and then dried *in vacuo*.

Using PE immuno-sticks as the substrate, PS grafting to an extent of ca. 15 wt. % was obtained. Irradiation of low-density polyethylene film (thickness 54 μm) under the same conditions resulted in ca. 250 wt % grafting. This apparently very great difference is, as discussed in Example 12 (*vide infra*), merely a consequence of the much greater thickness of the PE material of the immuno-sticks than that of the film, the surface density of grafting for the two types of PE substrate being comparable.

EXAMPLE 2

(a) *Procedure for grafting on non-woven felt made from fibers consisting of a polypropylene core and another layer of high-density polyethylene.*

The non-woven PP/PE felt was washed with n-hexane and irradiated in a closed ampoule containing a degassed 30% (v/v) solution of purified styrene in methanol in a manner completely analogous to the general procedure described in Example 1(a). The results are given in Table 2(a).

TABLE 2(a)

Grafting of PP/PE non-woven felt in methanol/styrene (70:30 v/v)

Mass of PP/PE felt (g)	Irradiation dose (kGy) (Irradiation time in hours)	Graft %*	Duration of extraction with CH_2Cl_2 (hours)
0.2400	1.6 (4.5)	86	124
0.2084	2.1 (6.0)	106	72

* : Graft % =
[(mass of final sheet) -(mass of polyethylene)] x 100/(mass of polyethylene)

(b) *Alternative procedure for grafting on non-woven felt made from fibers consisting of a polypropylene core and another layer of high-density polyethylene.*

The non-woven PP/PE felt was irradiated in a closed ampoule containing a degassed 30% (v/v) solution of purified styrene in methanol in a manner completely analogous to the procedure described in Example 1(b). Specific data for the preparation of a series of polystyrene-grafted PP/PE felt samples are given in Table 2(b).

TABLE 2(b)

Grafting of PP/PE non-woven felt in methanol/styrene (70:30 v/v)

Mass of PP/PE felt (g)	Irradiation dose (kGy)	Graft %*
0.24	ca. 1.5	ca. 50
0.21	ca. 2.1	ca. 100

EXAMPLE 3

Influence of methanol/styrene ratio on grafted polystyrene chain length.

The following results were obtained for irradiation of low-density polyethylene sheets in different methanol/styrene mixtures (5 kGy dose, 400 Gy/hour dose rate, room temperature):

% Methanol in solvent (v/v)	Peak molecular weight of homopolymer
0	180,000
20	300,000
40	500,000
70	800,000

It is seen that the molecular weight (determined by size-exclusion chromatography on cross-linked styrene/divinylbenzene column material) of the homopolymer fraction occluded amongst the grafted

chains of the polystyrene-grafted polyethylene sheets and extracted from the sheets with dichloromethane increases as a function of the methanol/styrene ratio in the solution. At the same time the molecular weight distribution tends to become more narrow for high methanol/styrene ratios.

EXAMPLE 4

Experiments on the estimation of the molecular weight of the grafted polystyrene chains.

The polystyrene homopolymer extracted from the sheets was characterized by SEC. The extracted polystyrene shows typically a molecular weight distribution with two bulges. This is due to the fact that polystyrene is formed both in the sheet and in the surrounding solution during irradiation. If a sheet is washed briefly in dichloromethane before carrying out Soxhlet extraction, the amount of low molecular weight fraction is greatly reduced relative to that of the high molecular weight fraction. Molecular weight data for the high molecular weight fraction of the extracted homopolymer are given in Table 3. Typical sample sizes were from 0.01 mg to 0.2 mg of polymer. For the homopolymer from the sheets grafted to the extent of 173, 220, 231 and 450 wt %, respectively, a 60 cm column of Toyo Soda TSK GMH6 was used for SEC at ambient temperature with THF as the eluent and a flow rate of 0.5 ml/min. For the homopolymer from the sheet grafted to the extent of 443 wt %, a 50 cm column of Shodex A80-M was used for SEC at 50°C with xylene as the solvent and a flow rate of 0.5 ml/min. This set-up was also used for SEC of the grafted polymer formed, but now operating at 90°C and with a flow rate of approximately 0.3 ml/min. The grafted sheets are soluble in hot xylene. Molecular weight data for polystyrene-grafted polyethylene from the five grafted sheets are given in Table 4. All molecular weight data given were calculated using a calibration curve based on polystyrene standards with molecular weights from 2800 g/mol to 8,000,000 g/mol, the form of the calibration curve being fitted by a third-order polynomial. The use of a first-order (linear) calibration curve leads to similar results. It should be noted that whereas the

molecular weights obtained for the styrene homopolymer are absolute, the molecular weights obtained for the graft copolymer are not absolute. In the case of the Shodex A80-M column, extrapolation of the calibration curve was necessary in order to calculate the extremely high molecular weights observed. This extrapolation may lead to underestimation of the weight average molecular weight and consequently also of the polydispersity. The ungrafted polyethylene was characterized by high-temperature SEC using 1,2,4-trichlorobenzene as the eluent. The following values were obtained: $M_w = 4 \times 10^4$ g/mol and $M_w/M_n = 5$. The latter data were obtained using a calibration curve based on polystyrene standards.

The data for the polystyrene homopolymers indicate that the molecular weight is insensitive to the total radiation dose, whereas for the polystyrene-grafted polyethylene the measured molecular weight is largely proportional to the dose. These observations indicate that the very long chain grafts are formed during the entire irradiation process and that essentially only the number of grafts is affected by the dose.

TABLE 3

Molecular weight data for the high molecular weight fraction of the polystyrene homopolymer extracted from the irradiated sheets

Graft %	Irradiation dose (kGy)	M_w# (x10^{-6} mol/g)	M_w/M_n§
450	3.4	1.4	2.2
443	4.0	2.6	3.7
231	2.7	1.2	2.3
220	3.0	1.2	2.2
173	2.4	1.1	2.2

: M_w - weight average molecular weight

§ : M_w/M_n - weight average molecular weight divided by number average molecular weight

TABLE 4

Molecular weight data for the polystyrene-grafted polyethylene from the irradiated sheets.

Graft %	Irradiation dose (kGy)	M_w# (x10^{-6} mol/g)	M_w/M_n§	M_{peak}* (x10^{-6} mol/g)
450	3.4	7.0	1.6	7.2
443	4.0	6.6	1.8	6.1
231	2.7	2.9	2.2	3.5
220	3.0	4.7	1.6	4.5
173	2.4	4.1	1.6	3.1

# : M_w	=	weight average molecular weight
§ : M_w/M_n	=	weight average molecular weight divided by number average molecular weight
* : M_{peak}	=	molecular weight at the peak point in the chromatogram

EXAMPLE 5

(a) *Reshaping of a polystyrene-grafted polyethylene sheet.*

A piece of 173% graft sheet (cf. Table 1) was dissolved in xylene at 100°C and the solution poured into a teflon mold at 80°C. After slow evaporation of the xylene a very thin film was formed. Because of the extreme thinness of the film it was not possible to obtain anything but small pieces (1 to 2 mm square) of film. However, these small pieces did not disintegrate when exposed to dichloromethane. This implies that a continuous polyethylene phase is reformed.

(b) *Surface coating of glass plates with a film of polystyrene-grafted polyethylene.*

A piece of polystyrene-grafted polyethylene sheet was dissolved in toluene at 90°C and the solution was poured onto a glass plate at 90°C. After slow evaporation of the toluene, the glass plate was seen to be covered by a thin film of polystyrene-grafted polyethylene.

(c) *Surface coating of poly(methylpentene) plates with a film of polystyrene-grafted polyethylene.*

Using a procedure analogous to that in (b), above, plates of poly(methylpentene) could be coated with a thin film of polystyrene-grafted polyethylene.

EXAMPLE 6

Aminomethylation (functionalization) of polystyrene-grafted PE sheets.

Eight equally sized rectangular strips (1.5 x 4.5 cm) of 443% polystyrene-grafted polyethylene sheet (see Example 1(a)) (1.30 g total) were placed in a 60 ml SPPS reaction vessel on a manual SPPS shaker and washed with 40 ml of TFA/CH_2Cl_2 (1:1 v/v) for 3 x 5 min. A solution of 0.35 g (1.9 mmol) *N*-(hydroxymethyl)phthalimide (97% purity; EGA-CHEMIE) in 40 ml of TFA/CH_2Cl_2 (1:1 v/v) was added to the washed sheets and the mixture was shaken for 10 min. 10 ml of TFMSA/TFA/CH_2Cl_2 (10:45:45 v/v/v) was added slowly over a 4-5 hour period and shaking was continued for another 3 hours. The sheets were isolated by filtration and washed sequentially with the following: TFA/CH_2Cl_2 (1:1 v/v) (120 ml), CH_2Cl_2 (240 ml), methanol (160 ml), and ethanol (160 ml). They were then shaken in 40 ml of ethanol containing 10% of hydrazine (Fluka) for 12 hours at 70°C. The sheets were filtered from the hot mixture and washed sequentially (with 20 min shaking for each wash) with the following: hot ethanol (3 x 40 ml), hot DMF (3 x 40 ml), hot ethanol (3 x 40 ml), hot methanol (3 x 40 ml), and CH_2Cl_2 (3 x 40 ml). Finally, the sheets were treated with 40 ml of DIEA/CH_2Cl_2 (1:9 v/v) for 2 x 5 min, washed with 200 ml of CH_2Cl_2, and dried at room temperature. A total of 4 spectrophotometric ninhydrin colour tests ("Kaiser test") indicated 1.00 mmol NH_2/g sheet (0.99, 0.96, 1.02, and 1.01 mmol/g, respectively), and elemental analysis indicated 1.07 mmol N/g sheet. The following polystyrene-grafted polyethylene sheets have also been aminomethylated:

331% grafted sheet: substitution = 0.21 mmol NH_2/g sheet.
547% " " " " " 0.46 " " "
129% " " " " " 0.50 " " "
46% " " " " " 0.02 " " "
285% " " " " " 0.6 " " "

Grafted films prepared as in Example 1(b) were also aminomethylated

in an analogous manner, substitution ranging from 0.01 to 4.00 mmol NH_2/g film being found.

EXAMPLE 7

Preparation of BocGln-4-(oxymethyl)-Pam-sheet.

0.63 g aminomethyl-sheet (substitution = 1.0 mmol/g sheet; 443% graft) was pre-washed in 30 ml DMF/CH_2Cl_2 (1:2 v/v) for 3 x 3 min in a 60 ml reaction vessel on a SPPS shaker. 0.98 g Boc-Gln-4-(oxymethyl)phenylacetic acid (2.5 mmol, 4 equiv.) and 0.38 g HOBt (2.5 mmol, 4 equiv.) were dissolved in 20 ml DMF/CH_2Cl_2 (1:1 v/v) and stirred in a screw-capped tube for 3 min at 0°C. 0.52 g DCC was dissolved in 10 ml CH_2Cl_2 and added to the mixture. After stirring for 25 min at 0°C, DCU was filtered off and the filtrate was added to the pre-washed aminomethyl-sheet and shaken for 2 h. The sheets were filtered, washed with CH_2Cl_2, neutralized with DIEA/CH_2Cl_2 (5:95 v/v), washed with CH_2Cl_2, and dried. The absence of positive ninhydrin tests indicated quantitative coupling, which was also confirmed after removal of the Boc group by the following treatment: 30 ml TFA/CH_2Cl_2 (1:1 v/v) for 1 x 2 min and 1 x 30 min, 30 ml CH_2Cl_2 for 6 x 1 min, 30 ml DIEA/CH_2Cl_2 (5:95 v/v) for 2 x 5 min, and 30 ml CH_2Cl_2 for 4 x 1 min. 2 ninhydrin tests then indicated the extent of -NH_2 substitution to be 0.76 mmol NH_2/g sheet (0.74 and 0.77 mmol/g, respectively), which is very close to the theoretical value of 0.78 mmol NH_2/g sheet.

EXAMPLE 8

Peptide Synthesis: (a) Assembly of Protected Human [Asp^{76}]-Parathyroid Hormone Fragments 80-84, 75-84 and 70-84 on 443 wt % Polystyrene-grafted Polyethylene Sheet.

BocGln-OCH_2-Pam-sheet (0.80 g, 443% graft, 0.58 mmol Gln) was placed in a 60 ml reaction vessel on a SPPS shaker. Protected hPTH 70-84, (see Fig. 1) was assembled using the following synthetic protocol:

(1) CH_2Cl_2, 35 ml, 3x1 min;
(2) TFA/CH_2Cl_2 (1:1 v/v), 35 ml, 3x1 min;
(3) TFA/CH_2Cl_2 (1:1 v/v), 35 ml, 30 min;
(4) CH_2Cl_2, 35 ml, 6x1 min;
(5) DIEA/CH_2Cl_2 (1:19 v/v), 35 ml, 3x2 min;
(6) CH_2Cl_2, 35 ml, 6x1 min;
(7) 3 to 10 mg samples were cut off for ninhydrin analysis;
(8) protected amino acid was coupled as pre-formed symmetric anhydride (3 equiv., 0.05 M) in 35 ml DMF/CH_2Cl_2 (1:4 v/v), with shaking for 2 hours;
(9) CH_2Cl_2, 35 ml, 4x2 min;
(10) 3 to 10 mg samples were cut off and neutralized by repeating (5) and (6) before ninhydrin analysis.

All couplings were single couplings. Monitoring of the synthesis by using the quantitative ninhydrin test [originally developed for peptide synthesis on beads; see e.g. Sarin et al., Anal. Biochem., *117*, 147 (1981)] was successfully applied (Table 5), and with the exception (for unknown reasons) of the result for the second amino acid coupling (i.e. with formation of Boc-Ser^{83}(Bzl)Gln^{84}-OCH_2-Pam-sheet), indicated satisfactory values for the coupling efficiency in each coupling step.

TABLE 5

Quantitative ninhydrin monitoring[a] of the solid-phase synthesis of protected human parathyroid hormone fragment (70-84) on 443 wt % polystyrene-grafted polyethylene

		Coupling[b]		Deprotection	
Residue coupled		Remaining free amino groups (μmol/g)	Estimated[c] % completion	Measured substitution (mmol/g)	Theoretical substitution (mmol/g)
84	BocGlnX[d]	0.0	100	0.76 ± 0.02	0.78
83	BocSer(Bzl)	38.0	94.0	0.35 ± 0.04	0.69
82	BocLys(2Cl-Z)	1.6	99.7	0.54 ± 0.03	0.57
81	BocAla	0.6	99.9	0.52 ± 0.03	0.55
80	BocLys(2Cl-Z)	1.2	99.7	0.53 ± 0.02	0.47
79	BocThr(Bzl)	0.0	100	0.44 ± 0.03	0.43
78	BocLeu	0.4	99.9	0.39 ± 0.01	0.41
77	BocVal	0.0	100	0.39 ± 0.03	0.40
76	BocAsp(OBzl)	0.0	100	0.35 ± 0.01	0.37
75	BocVal	0.2	99.9	0.31 ± 0.02	0.35
74	BocAsp(OBzl)	0.7	99.8	0.31 ± 0.02	0.33
73	BocAla	0.0	100	0.29 ± 0.01	0.33
72	BocLys(2Cl-Z)	1.1	99.6	0.30 ± 0.02	0.30
71	BocAsp(OBzl)	1.3	99.5	0.28 ± 0.02	0.28
70	BocAla	0.0	100	0.23 ± 0.01	0.27

[a] Average values based on 2-4 ninhydrin analyses after coupling and deprotection in each cycle expressed as mmol/g of peptide-sheet.

[b] No residues were recoupled, but coupling of Boc-Ser(Bzl) was followed by complete acetylation of remaining free amino groups using N-acetylimidazole in methylene chloride.

[c] These estimated values are calculated relative to the theoretical substitution after coupling of the Boc-protected residue, and do not include correction for incomplete coupling of the preceding residue.

[d] X = $-OCH_2-C_6H_4-CH_2-COOH$.

(b) Cleavage, purification and identification of synthetic hPTH-(80-84).

90 mg of H-Lys(ClZ)AlaLys(ClZ)Ser(Bzl)GlnOCH$_2$-Pam-sheet was treated with 5 ml of anhydrous HF/anisole (9:1 v/v) for 1 h at 0°C to simultaneously deprotect the side-chain protecting groups and cleave the peptide from the sheet. Extractions with ether, to remove organic components such as anisole and alkylated anisoles, were followed by extraction into 10% aqueous acetic acid. Lyophilization gave 24.0 mg of crude product of high purity [see HPLC chromatogram in Fig. 2 (A)].

The crude product was purified in two steps on a preparative C_{18} column (300 x 19 mm). Buffers including TFA were evaporated off under reduced pressure and the product was redissolved in water; the solution was filtered and lyophilized to give 17.8 mg of H-LysAlaLys-SerGln-OH, as confirmed by amino acid analysis (Table 6) and FABMS molecular weight measurements (Table 7).

The overall synthetic yield was approx. 84%, and the yield of pure peptide was approx. 69% based on the quantitative amino acid analysis.

(c) Cleavage, purification and identification of synthetic hPTH-(75-84).

93 mg of H-ValAsp(OBzl)ValLeuThr(Bzl)Lys(ClZ)AlaLys(ClZ)Ser(Bzl)Gln--OCH$_2$-Pam-sheet was treated in the same manner as for hPTH-(80-84), giving 36.6 mg of crude product [see HPLC chromatogram in Fig. 2 (B)], and finally 27.2 mg of purified peptide, identified by amino acid analysis (Table 6) and molecular weight measurements (Table 7). The overall synthetic yield was approx. 85%, and the yield of pure peptide was approx. 69%, based on the quantitative amino acid analysis.

(d) Cleavage, purification and identification of synthetic hPTH-(70-84).

The hPTH-(70-84) fragment was released from 96 mg of peptide-sheet in the same way as in (*b*) and (c), above [see HPLC chromatogram in Fig. 2 (C)]. The overall synthetic yield was approx. 83% and the yield of pure peptide was 26 mg (approx. 63%). The pure peptide was identified by amino acid analysis (Table 6) and molecular weight measurements (Table 7).

TABLE 6

Amino acid composition of purified synthetic hPTH compounds

Amino acid	Molar ratio[a]		
	hPTH-(70-84)	hPTH-(75-84)	hPTH-(80-84)
Asp	2.94 (3)	1.01 (1)	-
Thr[b]	0.99 (1)	0.95 (1)	-
Ser[b]	1.05 (1)	0.81 (1)	0.94 (1)
Glu	1.31 (1)	1.03 (1)	1.07 (1)
Ala	3.00 (3)	1.00 (1)	1.00 (1)
Val	2.00 (2)	2.00 (2)	-
Leu	1.03 (1)	1.00 (1)	-
Lys	3.08 (3)	1.98 (2)	1.89 (2)

Hydrolyses were performed in sealed, evacuated tubes with 5.7 M HCl containing 0.05 % phenol, 110°C, 20 h. Analysis by HPLC using fluorescence detection (338/450 nm) following treatment (post-column) with an *o*-phthaldialdehyde derivatizing reagent.

[a] Values in parentheses are theoretical.

[b] Thr and Ser values were not corrected for loss during hydrolysis.

TABLE 7

Molecular weights of hPTH compounds

Peptide	Molecular weight	
	measured	calculated
hPTH-(80-84)	560.3	560.3
hPTH-(75-84)	1088	1088
hPTH-(70-84)	1588	1588

Determined by quadrupole mass spectrometry. The calculated m/z values are for C = 12.000 u and H = 1.008 u.

EXAMPLE 9

Rapid parallel synthesis of multiple peptide analogs:

(a) Labeling of sheets

Aminomethylated 285 wt % polystyrene-grafted polyethylene sheet (0.6 mmol NH_2/g sheet) was cut into thirteen discrete pieces (each piece: 1.5 x 3 cm, ca. 50 μm thickness, ca. 40 mg) and labeled individually by means of graphite-based ink. A piece of polyethylene film was placed on top of each labeled surface and melted into the grafted sheet by using an electrically heated sealing apparatus. Finally, the sheets were shaken in 50% TFA/CH_2Cl_2 for 20 min. to check that all labels were adequately sealed in.

(b) Simultaneous synthesis of melittin-(7-21) and twelve analogs on labeled sheets

Protected melittin-(7-21), i.e.

Boc-Lys(ClZ)-Val-Leu-Thr(Bzl)-Thr(Bzl)-Gly-Leu-Pro-Ala-Leu-Ile-Ser(Bzl)-Trp(CHO)-Ile-Lys(ClZ)-Pam-sheet,

and twelve analogs derived by substitutions in positions 12 and 14 (sequences of the free peptides are listed in Fig. 3) were each assembled stepwise on a labeled sheet. The common steps of deprotection, neutralization, washing and coupling of identical amino acids were performed simultaneously in a single reaction vessel, while the coupling of different amino acids was carried out in separate vessels.

A standard solid-phase procedure was employed, using double DCC coupling (3.5 equiv., 0.05 M, in 30% DMF/CH_2Cl_2) of all residues except for Boc-Gln and Boc-Asn, which were double coupled as HOBt esters in 30% DMF/CH_2Cl_2, and Boc-Leu^{13} to Gln^{14}, which was double coupled as a symmetric anhydride in 20% DMF/CH_2Cl_2.

Following removal of the N-terminal Boc group, deprotection and release of the peptides from the sheets were accomplished by the low/high HF method (Tam et al., *J. Am. Chem. Soc.*, 105, 6442 (1983)). The free 15-residue peptides were obtained in overall synthetic yields of ca. 70%. Fig. 4 shows HPLC chromatograms of the thirteen unpurified peptides. All peptides were purified in 1-2 steps on a semi-preparative C_{18} column. As an example, 3.2 mg of pure melittin-(7-21) was obtained from 1 cm^2 (23.2 mg) of fully protected peptide-sheet. The identity of the peptides was verified by amino acid analysis (Table 8) and molecular weight measurements (Table 9).

TABLE 8

Amino acid composition of purified melittin-(7-21) and its analogs

Amino acid	Molar ratio[a]				
	pept. 1	pept. 2	pept. 3	pept. 4	pept. 5
Lys	1.95 (2)	1.69 (2)	1.77 (2)	2.83 (3)	1.47 (2)
Val	0.65 (1)	0.82 (1)	0.87 (1)	0.78 (1)	0.87 (1)
Leu	2.97 (3)	2.85 (3)	2.94 (3)	2.67 (3)	2.86 (3)
Thr[b]	1.87 (2)	1.89 (2)	1.93 (2)	1.70 (2)	1.84 (2)
Gly	1.08 (1)	1.03 (1)	2.04 (2)	1.07 (1)	1.12 (1)
Pro	0.98 (1)	0.98 (1)	-	-	-
Ala	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Ile	1.93 (2)	1.75 (2)	1.81 (2)	1.75 (2)	1.77 (2)
Ser[b]	0.90 (1)	0.96 (1)	0.97 (1)	1.00 (1)	0.98 (1)
Trp[c]					
Asp	-	-	-	-	(1)

	pept. 6	pept. 7	pept. 8	pept. 9	pept. 10[d]
Lys	1.87 (2)	1.71 (2)	1.83 (2)	1.93 (3)	(2)
Val	0.88 (1)	0.81 (1)	0.88 (1)	0.87 (1)	(1)
Leu	2.92 (3)	2.93 (3)	3.90 (4)	3.06 (3)	(3)
Thr[b]	1.87 (2)	1.90 (2)	1.88 (2)	2.06 (2)	(2)
Gly	1.09 (1)	1.08 (1)	1.05 (1)	1.26 (1)	(1)
Ala	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	(1)
Ile	1.82 (2)	1.80 (2)	1.83 (2)	1.95 (2)	(2)
Ser[b]	0.95 (1)	1.88 (2)	0.95 (1)	1.01 (1)	(1)
Trp[c]					
Asp	1.04 (1)	-	-	-	
Phe	-	-	-	0.91 (1)	(1)

	pept. 11	pept. 12	pept. 13
Lys	1.75 (2)	1.66 (2)	2.62 (3)
Val	1.75 (2)	0.81 (1)	0.89 (1)
Leu	2.73 (3)	3.70 (4)	3.71 (4)
Thr[b]	1.92 (2)	1.62 (2)	1.93 (2)
Gly	1.05 (1)	-	-
Ala	1.00 (1)	1.00 (1)	1.00 (1)
Ile	1.73 (2)	1.73 (2)	1.80 (2)
Ser[b]	0.97 (1)	1.00 (1)	0.95 (1)
Trp[c]			
Glu	-	1.12 (1)	-

The peptides were hydrolyzed in 5.7 M HCl at 110°C for 18 h in sealed non-evacuated tubes, except peptide 1 which was hydrolyzed for 24 h in an evacuated tube. After filtration, hydrolysates were analyzed on a Beckman 6300 amino acid analyzer.

[a] Values in parentheses are theoretical.

[b] Thr and Ser values were not corrected for loss during hydrolysis.

[c] Tryptophan was not determined.

[d] Peptide analog number 10 was not analyzed.

TABLE 9

Molecular weights of melittin-(7-21) analogs[a]

Peptide	Molecular weight		
	measured	calculated	Δ[b]
1	1640.5	1640.0	+0.5
2	1640.1	1640.0	+0.1
3	1600.0	1599.9	+0.1
4	1671.2	1671.1	+0.1
5	1658.0	1658.0	0.0
6	1657.0	1657.0	0.0
7	1630.2	1630.0	+0.2
8	1656.1	1656.0	+0.1
9	1690.0	1690.1	-0.1
10	1690.3	1690.1	+0.2
11	1642.2	1642.0	+0.2
12	1727.4	1727.1	+0.3
13	1727.3	1727.2	+0.1

[a] Determined by ^{252}Cf fission fragment time of flight mass spectrometry from the mean of the most abundant isotopes.

[b] Δ = measured - calculated.

EXAMPLE 10

The following example illustrates the efficiency of peptide synthesis carried out on aminomethylated, polystyrene-grafted polyethylene film using the Fmoc strategy of N-protection, instead of the Boc strategy, in the coupling of the individual amino acids.

Synthesis of H-Phe-Leu-Glu-Glu-Val-OH

A grafted film (0.089 g) grafted to the extent of 86% and aminomethylated as described above so as to have a substitution of 0.1 mmol NH_2/g was cut into 10 pieces (ca. 4 x 10 mm) and placed in the reactor column of a fully automatic, monitored, recirculating peptide synthesizer. After recirculation with DMF for 20 min., 4-hydroxymethylphenoxyacetic acid Dhbt ester (0.089 mg in ca. 1 ml DMF, i.e. a ca. 0.1 molar solution) was added and left with recirculation overnight. The initial yellow colour gradually faded, and the film became almost colourless. After washing with DMF and ethanol according to the synthesis program, the film was colourless.

The first amino acid was added as the anhydride: Fmoc-Val-OH (34 mg [0.1 x 339 g/mol]) was dissolved in dichloromethane (2 ml), cooled to 0°C, and diisopropylcarbodiimide (7.7 µl [0.05 x 126 g/mol]; density = 0.815) was added. The mixture was left for 10 min. After evaporation of the solvent *in vacuo*, the mixture was dissolved in DMF (a small amount taken from the column) and added to the column containing the film. 4-(Dimethylamino)pyridine (1.2 mg [0.01 x 122 g/mol]) was added as catalyst, and the mixture was recirculated for 90 min., followed by washing with DMF and ethanol.

The following amino acids were added as the Fmoc-protected and carboxylic acid activated Dhbt esters and recirculated in DMF; after acylation in the single steps had been accomplished, the films were washed with DMF and ethanol:

Fmoc-Glu(tBu)-Dhbt (57 mg);
Fmoc-Glu(tBu)-Dhbt (57 mg);

Fmoc-Leu-Dhbt (50 mg);
Fmoc-Phe-Dhbt (53 mg);

After completed synthesis the films were washed with ether and dried. Weight: 89.5 mg.

5 mg of film was treated with 95% TFA (0.75 ml) for 2 hours, the film was washed with additional TFA (3 x 0.3 ml), and the combined solutions were evaporated to dryness to give 0.4 mg of peptide. Amino acid analysis: Glu 1.99; Val 0.940; Leu 1.057; Phe 0.995.

EXAMPLE 11

Use of aminomethylated polystyrene-grafted polyethylene sheet as a support for the synthesis and ELISA detection of antigenic peptides.

The peptides assembled and tested were angiotensin II, i.e.:

H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH,

and HIV-2 peptide, i.e.:

H-Leu-Asn-Ser-Trp-Gly-Cys-Ala-Phe-Arg-Gln-Val-Cys-OH

Synthesis: The *in situ* syntheses of the latter peptides were carried out using the Fmoc strategy in a manner completely analogous to that illustrated in Example 10, but without the inclusion of the 4-hydroxymethylphenoxyacetic acid linker. The peptides thus remained firmly attached to the support.

Antisera: Anti-angiotensin II antiserum production in rabbits was performed by coupling angiotensin to the thyroglobulin carrier (Sofreniev et al., *Frezenius Z. Anal. Chem. 290* (1978) 163).

Human serum reactive against the HIV-2 peptide was from a confirmed HIV-2 seropositive patient. Normal human serum was from a healthy, anti-HIV-1 and anti-HIV-2 negative subject.

ELISA procedure: Uniformly sized pieces of the peptide-bearing sheets were washed four times in 0.1 M NaH_2PO_4/Na_2HPO_4 (pH 7.4), 0.15 M NaCl, 1% (w/v) Tween 20, 2% (w/v) skimmed milk powder (buffer A) for 40 min. at room temperature on a rocking table. They were then washed four times and incubated at room temperature for 1.5 hours with antisera diluted in buffer A (human anti-HIV-2 serum and normal human serum were diluted 1/50 to 1/400. Rabbit anti-angiotensin II serum and preimmunisation serum were diluted 1/100 to 1/32000 and 1/100 to 1/2000, respectively).

The sheets were washed four times and incubated for 45 min. on a rocking table at room temperature with peroxidase-labelled anti-antibodies (anti-human IgG and anti-rabbit IgG, respectively; DAKOPATTS, Denmark) diluted 1/3000 in buffer A. The sheets were washed four times, and colour development (10 minutes) was achieved with *o*-phenylenediamine (0.5 mg/ml) in citrate buffer, pH 5.0 with 0.05% H_2O_2. Colour development was stopped by adding 150 µl of 1 N H_2SO_4. The colour intensity as a function of serum dilution was assessed visually. The results indicated that the peptide-bearing substrates prepared as described were well-suited to a procedure of the ELISA type.

EXAMPLE 12

Use of aminomethylated polystyrene-grafted polyethylene "immuno-sticks" as support for the synthesis and ELISA detection of the antigenic peptide angiotensin II.

The following example supplements the preceding example and illustrates the use of a polystyrene-grafted polyethylene substrate in the form of a so-called "immuno-stick", i.e. a small rod (of square cross-section) at one end of which there are fashioned four mutually perpendicular blades or wings, two of which (two in the same plane) are slightly thicker than the other two, and which project outwards from the rod, the planes of the blades or wings being parallel with the longitudinal axis of the rod. The other end of the

rod is attached to the underside of an externally threaded stopper which is adapted for screwing into the correspondingly internally threaded neck of an associated (normally round-bottomed) cylindrical container (the stopper part of the immuno-stick and the cylindrical container suitably being made of polypropylene in the case of PE immuno-sticks to be used as described herein) into which the immuno-stick may be inserted. The polyethylene immuno-sticks employed here were made of high-density polyethylene and were fabricated specially for the purposes of experiments performed in connection with the present invention; these particular immuno-sticks are not presently commercially available, but their form and dimensions were essentially identical with those of commercially available polystyrene products marketed under the names Nunc-Immuno® Stick MaxiSorp and Nunc-Immuno® Stick PolySorp and available from Nunc A/S, DK-4000, Roskilde, Denmark under the catalogue numbers 472230 and 475574, respectively.

For the experiments described here, polyethylene immuno-sticks grafted as described in Example 1(c) with polystyrene to an extent of ca. 13% were employed. However, in this connection it should be noted that the surface density of grafting (i.e. number of grafts per unit surface area) for this particular substrate embodiment at this level of grafting is comparable to that for substrates in the form of films or sheets as employed in preceding examples and having a grafting percentage of the order of about 200%, the thickness of the polyethylene material of such immuno-sticks being considerably greater than that of the latter sheets or films.

Aminomethylation of the polystyrene-grafted PE immuno-sticks; measurement of the degree of amino group substitution: Amino-methylation of the PS-grafted PE immuno-sticks was carried out in the manner described previously, above.

The degree of amino group substitution of a thus-aminomethylated immuno-stick as determined directly by means of a spectrophotometric ninhydrin test (Kaiser test; *vide supra*) was found to be ca. 0.19 mmol NH_2 per g of PS grafted to PE, a value which is incompatible with the level of loading of attached (synthesized) peptide which

appears (on the basis of our results in general) to be attainable with this particular substrate embodiment. However, after coupling an amino acid (Boc-protected alanine with a Pam linker) to an aminomethylated immuno-stick via the attached aminomethyl groups, acylating any remaining unreacted aminomethyl groups, removing the Boc protecting groups and then performing a new Kaiser test to determine the level of substitution with alanine amino groups, a value of ca. 0.52 mmol NH_2/g PS was found. There are thus strong indications that the actual degree of aminomethylation in the case of this particular substrate embodiment is at least 2.5 times higher than determined by means of a Kaiser test performed directly on the aminomethylated PS-grafted PE immuno-sticks. This discrepancy is believed to be a consequence of the fact that ethanol, which is the solvent for the reagents employed in the Kaiser test, is too hydrophilic to be able to swell or solvate polystyrene or polyethylene to any large extent, so that access of the reagents in question to aminomethyl groups other than those lying fairly close to the outermost regions of the PS chains grafted to the PE material is restricted; the high-density nature of the PE in the immuno-sticks may also have some influence in this respect. The PE material of the films or sheets of the type employed in preceding examples is considerably thinner than the PE material of the immuno-sticks, and in practice it would appear that this permits more effective swelling of the PS-grafted thin PE films or sheets by ethanol than is the case for immuno-sticks. By coupling an amino acid (which is far more hydrophilic than PS or PE) to the aminomethyl groups, a more hydrophilic environment is obtained; thus, access by the reagents in question (dissolved in ethanol) to free, terminal amino groups of the coupled amino acids is expected to be considerably easier than access to free amino groups of aminomethyl groups attached directly to the PS chains.

Reagents and solvents employed in the synthesis: Amino acids [MilliGen PEPSYN reagents (as Fmoc-protected Pfp esters)]; DMF [Rein den Hahn (technical grade)], freshly redistilled before use; piperidine (Rein den Hahn), redistilled before use; HOBt [Fluka (>98%)]; Dhbt-OH (Aldrich); ethanol (96%); trifluoroacetic acid. For use in the individual amino acid coupling steps, solutions of the

respective amino acids (as Fmoc-protected Pfp esters) were made up in 3 ml of a 0.06M solution of HOBt in DMF, such that each solution was 0.3M with respect to the amino acid in question; this quantity of each amino acid solution is sufficient for three individual immuno-sticks.

Synthesis: As in the preceding example, the *in situ* synthesis of angiotensin II was carried out using the Fmoc strategy and without the inclusion of a cleavable linker; the peptide thus remained firmly attached to the support.

In the procedure described here, three identical immuno-sticks were treated simultaneously and uniformly. After initially allowing the aminomethylated, PS-grafted PE immuno-sticks to swell/solvate in DMF for 20 minutes, the procedure was as follows:

A) Each aminomethylated, PS-grafted PE immuno-stick was transferred to an individual polyethylene tube (volume ca. 2 ml) equipped with a screw cap, after which 1 ml of the solution of the first amino acid in the sequence was introduced into each tube, which was then closed with the screw cap and shaken mechanically for 30 minutes.

B) The three immuno-sticks were then transferred to a single 20 ml flask and washed (with mechanical agitation) as follows: (i) 2 X 3 min. washes with 15 ml aliquots of DMF, (ii) wash for 1 min. with 15 ml of 96% ethanol, (iii) wash for 3 min. with 15 ml of 96% ethanol, and (iv) 3 X 3 min. washes with 15 ml aliquots of DMF.

C) After completion of the washing procedure, the immuno-sticks were then agitated mechanically for 20 min. in a single 20 ml flask with 15 ml of a 20% (v/v) solution of piperidine in DMF in order to remove the Fmoc protecting groups.

D) The immuno-sticks were then washed in a single 20 ml flask as follows (with mechanical agitation): (i) wash for 20 min. with 15 ml of DMF, (ii) wash for 3 min. with 15 ml of DMF, (iii) wash for 1 min. with 15 ml of 96% ethanol, (iv) wash for 3 min. with 15 ml of 96% ethanol, and (v) *n* X 3 min. washes with 15 ml aliquots of DMF. The

number, *n*, of necessary washes in step (v) is normally from 5 to 7, washing being taken to be complete when the addition of Dhbt-OH to the washings from the last wash results in no visible yellow coloration of the washings.

This cycle of steps A)-D) was then repeated for the second, third,...eighth amino acids in the sequence, using a new polyethylene tube in each amino acid coupling step for each of the three immuno-sticks. Each immuno-stick was then transferred to an individual glass tube equipped with a stopper. 1 ml of a mixture of TFA, anisole and ethanediol (100:5:3) was then added to each tube (to remove side-chain protecting groups); after 10 hours the liquid phase was removed by decantation and each immuno-stick was subjected to 3 X 5 min. washes with 1 ml aliquots of 95% TFA. Finally, the immuno-sticks were washed with diethyl ether and dried in a stream of nitrogen.

ELISA procedure: For the purposes of the procedure described below, the four blades of a single immuno-stick bearing angiotensin II synthesized thereon in the above manner were cut from the immuno-stick and used as follows:

The four blades were gently agitated for 30 minutes at ambient temperature (ca. 21 °C) in 0.1 M NaH_2PO_4/Na_2HPO_4 (pH 7.4), 0.5 M NaCl, 0.1% (w/v) Tween 20 (buffer B) to which had further been added 5% (w/v) of skimmed milk powder. They were then washed six times in 0.1 M NaH_2PO_4/Na_2HPO_4, 0.15 M NaCl, 0.1% (w/v) Tween 20 (buffer C).

The blades were then incubated individually with different dilutions (1:10, 1:50, 1:100 and 1:1000) of rabbit anti-angiotensin II antiserum (cf. Example 11) in buffer B to which had been added 1% (w/v) of skimmed milk powder. This incubation took place with gentle agitation for 1 hour at ambient temperature, after which the blades were washed six times in buffer C.

The four blades were then incubated with a 1:1000 dilution of peroxidase-labelled anti-antibodies (anti-rabbit IgG; DAKOPATTS A/S, Denmark, code P217) in 1 ml of a medium consisting of buffer B to

which had been added 1% of skimmed milk powder and 5% of normal horse serum. This incubation took place with gentle agitation for 1 hour at ambient temperature, after which the blades were washed six times in buffer C.

Colour development was achieved by immersing each blade for 15 minutes in 100 µl of a solution of *o*-phenylenediamine (3.6 mg/ml) in citrate buffer (pH 5.0) containing 0.02% of H_2O_2. Colour development was stopped by adding 100 µl of 2M H_2SO_4 per blade. The absorbance at 490 nm (A_{490}) of the resulting coloured solutions was determined using an ImmunoReader™ (InterMed NJ-2000, Nunc, Denmark).The results are summarized in Table 10, below.

As controls were used the correspondingly treated blades cut from a PE immuno-stick on which had been synthesized (in a manner exactly analogous to that described above for the preparation of immuno-sticks having angiotensin II synthesized thereon) a "nonsense" dipeptide sequence, viz. His-Phe, which does not react with the anti-angiotensin II antiserum. The absorbance (A_{490}) measurements for the controls are likewise summarized in Table 10.

Table 10

ELISA-type assay results for angiotensin II synthesized on PS-grafted PE immuno-sticks

Anti-angiotensin II antiserum dilution	A_{490}	
	Angiotensin II	"Nonsense" control
1:10	0.444	0.172
1:50	0.374	0.072
1.100	0.197	0.111
1:1000	0.150	0.004

It is clear from the results in Table 10 that the octapeptide angiotensin II synthesized on and covalently attached to the immuno-

stick support is highly immunoreactive towards the antibodies present in the anti-angiotensin II antiserum, the A_{490} values for the octapeptide being much higher than those for the "nonsense" dipeptide His-Phe.

Reversibility of antibody binding to the synthesized peptide: Another very interesting result obtained in connection with the above-described experiments is that it proved possible, using a buffered solution of an anionic detergent, to bring about desorption of the immunochemically bound antibodies from the antigenic peptide synthesized on the immuno-stick support:

After completion of the ELISA procedure described above, the four blades bearing the synthesized angiotensin II to which the anti-angiotensin II antibodies/anti-antibodies were immunochemically bound were each treated for 1 hour at 80 °C with 500 µl of 1% sodium dodecylsulfate in 0.1 M NaH_2PO_4/Na_2HPO_4 (pH 7.4), 0.5 M NaCl. The blades were then washed six times with buffer C (*vide supra*) and agitated gently in buffer B (*vide supra*) to which had further been added 5% (w/v) of skimmed milk powder, after which they were washed again six times with buffer C.

Two of the blades were incubated for 1 hour at ambient temperature in a 1:50 dilution of rabbit anti-angiotensin II antiserum (*vide supra*) in buffer B to which had been added 1% (w/v) of skimmed milk powder. This incubation took place with gentle agitation for 1 hour at ambient temperature, after which the blades were washed six times in buffer C. The other two blades (to be used as controls) were incubated for 1 hour at ambient temperature in buffer B to which had been added 1% (w/v) of skimmed milk powder and then washed six times in buffer C.

All four blades were then incubated with a 1:500 dilution of peroxidase-labelled anti-antibodies (*vide supra*) in 1 ml of a medium consisting of buffer B to which had been added 1% of skimmed milk powder and 5% of normal horse serum; they were then washed and subjected to *o*-phenylenediamine colour development in essentially the same manner as described above in connection with the ELISA

procedure. The absorbance (A_{490}) of a 200 µl aliquot of each solution transferred to a well of a Nunc-Immuno® Plate (Nunc A/S, Denmark) was then determined. The solutions deriving from the two blades subjected to treatment with the antibody dilution gave absorbance values of 1.054 and 1.181, respectively, whereas the solutions deriving from the two blades subjected only to treatment with buffer B + skimmed milk gave absorbance values of 0.307 and 0.192, respectively.

The origin of the large difference in the magnitudes of the absorbances measured here and in the case of the above-described ELISA procedure is not immediately apparent, and the experiments described in this section have not been optimized. However, it is clear from the latter results that the binding of antibody to the covalently anchored antigenic peptide (angiotensin II) may be reversed to some extent by the above-described treatment. Furthermore, there was no indication of any appreciable attendant degradation of the covalently anchored peptide. It thus appears that it may be possible, for example, to re-use a peptide-bearing support, such as an peptide-bearing immuno-stick support, for a number of assays.

EXAMPLE 13

Use of polystyrene-grafted polyethylene "immuno-sticks" with a cleavable linker as a support for the synthesis of angiotensin II.

The following example is included for the purpose of illustrating the suitability of PS-grafted PE immuno-sticks of the above described type may be used for the synthesis (for the purpose of subsequent isolation) of a representative peptide such as the octapeptide angiotensin II.

Reagents and solvents employed in the synthesis: 4-Dimethyl-aminopyridine (DMAP) (Fluka, cat. nr. 39405); N,N'-diisopropyl-carbodiimide (DIPCDI) (MilliGen, GEN 910006, batch 979); 4-(hydroxymethyl)phenoxyacetic acid Dhbt ester (HMPA-O-Dhbt)

(prepared by the inventors); dichloromethane (Merck, cat. nr. 6050), distilled once. Other reagents employed were as in Example 12.

Three PS-grafted PE immuno-sticks grafted as described in Example 1(c) to an extent of ca. 16% were employed and were aminomethylated as previously described. The HMPA linker was attached as follows:

1) After initially being subjected to swelling in DMF as described in Example 12, the sticks were screwed into immuno-stick tubes in which a solution of 245 mg of HMPA-O-Dhbt in 5 ml of DMF had been divided equally. The reaction was allowed to proceed for 1 hour with mechanical agitation, after which the reagent was removed by decantation.

2) The sticks were then washed as follows:
a: 2 X 3 minutes with 5 ml aliquots of DMF,
b: 1 min. with 5 ml of 96% ethanol,
c: 3 min. with 5 ml of 96% ethanol,
d: 3 min. with 5 ml of dichloromethane,
e: 3 X 3 min. with 5 ml aliquots of DMF.

3) For coupling of the first amino acid (Phe) to the linker, the Fmoc-protected amino acid was converted to the amino acid anhydride as follows: 691 mg was dissolved in 3 ml of dichloromethane and cooled to 0 °C (any tendency towards precipitation can be suppressed by the addition of a little DMF). 70 µl of DIPCDI was added, and the reaction was allowed to proceed for 10 minutes. The reaction mixture was then evaporated to dryness on a rotary evaporator, and the residue (containing the anhydride) was redissolved in 5 ml of DMF to which was added 18.3 mg of DMAP. The latter solution was divided among three new immuno-stick tubes, and the washed immuno-sticks were screwed into place in the tubes. The coupling reaction was allowed to proceed for 90 min. with mechanical agitation. The reagent solution was then removed and the immuno-sticks were washed as described i step 2).

4) Unreacted aminomethyl groups were then blocked by acetic acid

anhydride acylation. The immuno-sticks were then washed again as described in step 2).

5) Fmoc protecting groups were removed by allowing the immuno-sticks to stand in a 20% (v/v) solution of piperidine in DMF for 10 min., after which they were subjected to 10 X 1/2 min. washes with DMF and then washed as in step 2).

6) For coupling of the second, third, ...eighth amino acids in the sequence, the procedure for each amino acid was as follows:

3 ml of a 0.3 M solution of the amino acid in DMF containing 29.4 mg of Dhbt-OH was distributed equally between three immuno-stick tubes (so as to cover the blades of the immuno-sticks) and the reaction was allowed to proceed for 30 min. The immuno-sticks were then washed as in step 2). Removal of the Fmoc protecting groups and subsequent washing with DMF took place as described in step 5), after which coupling of the next amino acid could be carried out.

After completion of the synthesis, the peptide was cleaved from the immuno-stick supports using 95% TFA, from which the peptide could be isolated and further purified in the manner previously described. This treatment with TFA also removed side-chain protecting groups.

HPLC analysis of the product revealed it to be of high purity, and amino acid analysis after total hydrolysis showed the presence of the expected amino acids in the correct ratio.

EXAMPLE 14

Establishment of a functionalized coating of PS-grafted PE on a substrate material

This example describes briefly a procedure by which a PS substrate may be provided with a coating or layer of functionalized PS-grafted PE:

A *N*-phthalimidomethyl-substituted film resulting from the treatment of PS-grafted PE film (165% grafting) with *N*-(hydroxymethyl)-phthalimide in the manner described in Example 6 was dissolved in dichlorobenzene at 180 °C [concentration ca. 1% (w/w)], and a PS immuno-stick was then dipped quickly in the solution. After drying and cooling, the *N*-phthalimidomethyl groups on the resulting coating were converted to aminomethyl groups by treatment with 10% ethanolic hydrazine for 12 hours at 50 °C.

CLAIMS

1. A polymer substrate grafted with polystyrene chains, said polystyrene chains optionally further bearing substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate being functionalized with a chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein upon reaction of said functionality with an amino acid, a peptide or a protein.

2. A functionalized, polystyrene-grafted polymer substrate as claimed in claim 1, said amino acid, peptide or protein which is to react being (i) protected at the N-terminal and/or (ii) derivatized at the carboxyl terminal and/or, where relevant, (iii) side-chain protected.

3. A functionalized, polystyrene-grafted polymer substrate as claimed in claim 1 or 2, said peptide or protein being selected from the group consisting of:

peptide and protein antigens which participate in an *in vivo* and/or *in vitro* antigen/antibody reaction with an antibody,

antibodies which participate in an *in vivo* and/or *in vitro* antibody/antigen reaction with an antigen,

peptide and protein hormones, and

enzymes.

4. A functionalized, polystyrene-grafted polymer substrate as claimed in any of the preceding claims, wherein the estimated molecular weight of substantially all of the polystyrene chains

grafted to the polymer, not including optional substituents, is in the range of 300,000-1,600,000, preferably 400,000-1,400,000, more preferably 600,000-1,200,000, most preferably 700,000-1,000,000.

5. A functionalized, polystyrene-grafted polymer substrate as claimed in any of the preceding claims, which has been prepared from a polymer substrate in the form of a sheet or film of thickness in the range of 25 to 100 μm, and in which the degree of polystyrene chain grafting of the polymer substrate is in the range of 5-800% by weight, preferably 40-700%, more preferably 100-600%, most preferably 100-300%, especially 200-300% by weight.

6. A functionalized, polystyrene-grafted polymer substrate as claimed in any of the preceding claims, which is in the form of a sheet, film or net.

7. A functionalized, polystyrene-grafted polymer substrate as claimed in claim 6, which has a thickness of from 10 to 10,000 μm, preferably from 25 to 1000 μm, more preferably from 25 to 200 μm.

8. A functionalized, polystyrene-grafted polymer substrate as claimed in any of the preceding claims, wherein the chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between said amino acid, peptide or protein and the functionalized polystyrene moiety is a member of, or is derived from a member of the group consisting of:

chloro-, bromo- and iodo-substituted alkyl,

amino-substituted alkyl,

hydroxy-substituted alkyl,

the functionality, if derived from any of said group, being a functionality with a spacer group.

9. A functionalized, polystyrene-grafted polymer substrate as claimed in claim 8, wherein chloro-substituted alkyl is

chloromethyl, amino-substituted alkyl is aminomethyl, and hydroxy-substituted alkyl is hydroxymethyl.

10. A functionalized, polystyrene-grafted polymer substrate as claimed in claim 8 or 9, wherein the functionality is derived from an amino-substituted alkyl group, and the functionality comprises a spacer group derived from the group consisting of straight- and branched-chain ω-aminoalkanoic acids, preferably a spacer group derived from the group consisting of 6-aminohexanoic acid, 5-aminopentanoic acid, 4-aminobutanoic acid and 3-aminopropanoic acid.

11. A functionalized, polystyrene-grafted polymer substrate as claimed in any of the preceding claims, wherein the polymer is a polyolefin, preferably polyethylene.

12. A functionalized, polystyrene-grafted polymer substrate as claimed in any of the preceding claims and further supported on a substantially chemically inert and optionally light-transparent support material.

13. A supported, functionalized, polystyrene-grafted polymer substrate as claimed in claim 12, wherein said support material is selected from the group consisting of: glass, ceramics, poly(methylpentene), polytetrafluoroethylene, polypropylene, polyethylene, hard PVC, and silicone rubbers.

14. A supported, functionalized, polystyrene-grafted polymer substrate as claimed in claim 12 or 13 and fashioned in a form selected from the group consisting of a microtiter plate, a test tube, a beaker, a flask, a tray, a Petri dish, a strip, a rod and a fibre.

15. A polymer substrate grafted with polystyrene chains and to which a peptide or protein is coupled, said polystyrene-grafted substrate preferably being fashioned in a form selected from the group consisting of a microtiter plate, a test tube, a beaker, a flask, a tray, a Petri dish, a strip, a rod and a fibre, and being supported

on a substantially chemically inert and optionally light-transparent support material, said polystyrene chains optionally further bearing substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate bearing a substantially permanent covalent anchoring linkage via which said peptide or protein is coupled.

16. A supported, peptide- or protein-bearing polystyrene-grafted polymer substrate as claimed in claim 15, said peptide or protein being selected from the group consisting of:

peptide and protein antigens which participate in an *in vivo* and/or *in vitro* antigen/antibody reaction with an antibody,

antibodies which participate in an *in vivo* and/or *in vitro* antibody/antigen reaction with an antigen,

peptide and protein hormones, and

enzymes.

17. A supported, peptide- or protein-bearing polystyrene-grafted polymer substrate as claimed in claim 15 or 16, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being in the range of 300,000-1,600,000, preferably 400,000-1,400,000, more preferably 600,000-1,200,000, most preferably 700,000-1,000,000.

18. A supported, peptide- or protein-bearing polystyrene-grafted polymer substrate as claimed in any of claims 15 to 17, which has been prepared from a polymer substrate in the form of a sheet or film of thickness in the range of 25 to 100 μm, and in which the degree of polystyrene chain grafting of the polymer substrate is in the range of 5-800% by weight, preferably 40-700%, more preferably 100-600%, most preferably 100-300%, especially 200-300% by weight.

19. A supported, peptide- or protein-bearing polystyrene-grafted polymer substrate as claimed in any of claims 15 to 18, said peptide- or protein-bearing polymer substrate part having a thickness of from 10 to 10,000 μm, preferably from 25 to 1000 μm, more preferably from 25 to 200 μm.

20. A supported, peptide- or protein-bearing polystyrene-grafted polymer substrate as claimed in any of claims 15 to 19, wherein the polymer is a polyolefin, preferably polyethylene.

21. A supported, peptide- or protein-bearing polystyrene-grafted polymer substrate as claimed in any of claims 15 to 20, wherein said support material is selected from the group consisting of: glass, ceramics, poly(methylpentene), polytetrafluoroethylene, polypropylene, polyethylene, hard PVC, and silicone rubbers.

22. A method for the preparation of a polymer substrate grafted with polystyrene chains, said polystyrene chains optionally further bearing substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate being functionalized with a chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein upon reaction of said functionality with an amino acid, a peptide or a protein, the method comprising:

a) subjecting a polymer substrate immersed in a solution of optionally substituted styrene monomer in an organic solvent to a treatment leading to the formation of free radicals such that polystyrene chains which are grafted to the polymer substrate are formed,

b) washing the polystyrene-grafted polymer substrate produced according to step (a) with a suitable solvent or solvents so as to substantially completely remove non-grafted, optionally

substituted polystyrene chains and optionally substituted styrene monomer, and

c) functionalizing at least part of said grafted polystyrene chains with a chemical functionality facilitating the formation of an substantially permanent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein.

23. A method for the preparation of a polymer substrate grafted with polystyrene chains and supported on a substantially chemically inert and optionally light-transparent support material, said polystyrene chains optionally further bearing substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate being functionalized with a chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein upon reaction of said functionality with an amino acid, a peptide or a protein, the method comprising:

a) attaching or affixing a polymer substrate to said support material,

b) subjecting the supported polymer substrate immersed in a solution of optionally substituted styrene monomer in an organic solvent to a treatment leading to the formation of free radicals such that polystyrene chains which are grafted to the polymer substrate are formed,

c) washing the supported, polystyrene-grafted polymer substrate produced according to step (b) with a suitable solvent or solvents so as to substantially completely remove non-grafted, optionally substituted polystyrene chains and optionally substituted styrene monomer, and

d) functionalizing at least part of said grafted polystyrene chains with a chemical functionality facilitating the formation of an substantially permanent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein.

24. A method for the preparation of a polymer substrate grafted with polystyrene chains and supported on a substantially chemically inert and optionally light-transparent support material, said polystyrene chains optionally further bearing substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate being functionalized with a chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein upon reaction of said functionality with an amino acid, a peptide or a protein, the method comprising:

a) subjecting a polymer substrate immersed in a solution of optionally substituted styrene monomer in an organic solvent to a treatment leading to the formation of free radicals such that polystyrene chains which are grafted to the polymer substrate are formed,

b) washing the polystyrene-grafted polymer substrate produced according to step (a) with a suitable solvent or solvents so as to substantially completely remove non-grafted, optionally substituted polystyrene chains and optionally substituted styrene monomer,

c) attaching or affixing the polystyrene-grafted and washed polymer substrate to said support material, and

d) functionalizing at least part of said grafted polystyrene chains with a chemical functionality facilitating the formation of an substantially permanent anchoring linkage between the polystyrene moiety and an amino acid, a peptide or a protein.

25. A method as claimed in any of claims 22 to 24, said reacting amino acid, peptide or protein being (i) protected at the N-terminal and/or (ii) derivatized at the carboxyl terminal and/or, where relevant, (iii) side-chain protected.

26. A method as claimed in any of claims 22 to 25, said peptide or protein being selected from the group consisting of:

peptide and protein antigens which participate in an *in vivo* and/or *in vitro* antigen/antibody reaction with an antibody,

antibodies which participate in an *in vivo* and/or *in vitro* antibody/antigen reaction with an antigen,

peptide and protein hormones, and

enzymes.

27. A method as claimed in any of claims 22 to 26, wherein the treatment leading to the formation of free radicals is gamma irradiation.

28. A method as claimed in claim 27, wherein the gamma irradiation is performed using a gamma radiation dose rate of from about 1 to about 100,000 Gy/hour, preferably from about 200 to about 5,000 Gy/hour, more preferably from about 300 to about 1,000 Gy/hour.

29. A method as claimed in any of claims 22 to 28, wherein the organic solvent is an alcohol, preferably an aliphatic alcohol, more preferably a C_{1-4} aliphatic alcohol, most preferably methanol.

30. A method as claimed in any of claims 22 to 29, wherein the volume percentage (% v/v) of optionally substituted styrene in the solution is such that $0 < \%\ v/v < 100$, preferably such that $10 \leq \%\ v/v \leq 90$, more preferably such that $20 \leq \%\ v/v \leq 80$, most

preferably such that 25 ≤ % v/v ≤ 50, especially such that 25 ≤ % v/v ≤ 35.

31. A method as claimed in any of claims 22 to 30, wherein the grafting is performed so as to give an estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, in the range of 300,000-1,600,000, preferably 400,000-1,400,000, more preferably 600,000-1,200,000, most preferably 700,000-1,000,000.

32. A method as claimed in any of claims 22 to 31, wherein the polymer substrate is in the form of a sheet or film of thickness in the range of 25 to 100 μm, and the grafting is performed so as to give a degree of polystyrene chain grafting of the polymer substrate in the range of 5-800% by weight, preferably 40-700%, more preferably 100-600%, most preferably 100-300%, especially 200-300% by weight.

33. A method as claimed in any of claims 22 to 32, wherein the polymer substrate is in the form of a sheet or film.

34. A method as claimed in claim 33, wherein the polymer substrate has a thickness of from 10 to 10,000 μm, preferably from 25 to 1000 μm, more preferably from 25 to 100 μm.

35. A method as claimed in any of claims 22 to 34, wherein the chemical functionality facilitating the formation of a covalent anchoring linkage between said amino acid, peptide or protein and the functionalized polystyrene moiety is a member of, or is derived from a member of the group consisting of:

chloro-, bromo- and iodo-substituted alkyl,

amino-substituted alkyl,

hydroxy-substituted alkyl,

the functionality, if derived from any of said group, being a functionality with a spacer group.

36. A method as claimed in claim 35, wherein chloro-substituted alkyl is chloromethyl, amino-substituted alkyl is aminomethyl, and hydroxy-substituted alkyl is hydroxymethyl.

37. A method as claimed in claim 35 or 36, wherein the functionality is derived from an amino-substituted alkyl group, and the functionality comprises a spacer group derived from a member of the group consisting of straight- and branched-chain ω-aminoalkanoic acids, preferably a spacer group derived from a member of the group consisting of 6-aminohexanoic acid, 5-aminopentanoic acid, 4-aminobutanoic acid and 3-aminopropanoic acid.

38. A method as claimed in any of claims 22 to 37, wherein the polymer is a polyolefin, preferably polyethylene.

39. A method as claimed in any of claims 23 to 38, wherein said support material is selected from the group consisting of: glass, ceramics, poly(methylpentene), polytetrafluoroethylene, polypropylene, polyethylene, hard PVC, and silicone rubbers.

40. A method as claimed in any of claims 23 to 39, wherein said support material is fashioned in a form selected from the group consisting of a microtiter plate, a test tube, a beaker, a flask, a tray, a Petri dish, a strip, a rod and a fibre, the affixed, supported, polystyrene-grafted polymer substrate thereby acquiring said form.

41. A functionalized, polystyrene-grafted polymer substrate prepared by a method as claimed in any of claims 22 to 40.

42. A functionalized, polystyrene-grafted polymer substrate prepared by a method as claimed in any of claims 23 to 40.

43. A method for the preparation of a polystyrene-grafted polymer substrate bearing a peptide or protein thereon and supported on a substantially chemically inert and optionally light-transparent support material, the method comprising the steps of:

A) providing a supported polymer substrate grafted with polystyrene chains, said polystyrene chains optionally further bearing substituents which are not reactive under the conditions necessary to effect any of steps (B)-(G) below, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate being functionalized with a chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety and an at least *N*-protected and optionally carboxyl terminal derivatized amino acid or peptide, the functionalized, polystyrene-grafted polymer substrate being supported on a substantially chemically inert and optionally light-transparent support material,

B) coupling an *N*-protected and optionally carboxyl terminal derivatized amino acid or peptide to the functionalized polystyrene moiety,

C) in those cases where an amino acid has been coupled in step (B), and optionally in those cases where a peptide has been coupled in step (B), removing the *N*-protecting group from an *N*-protected amino or substituted amino group of the coupled and *N*-protected amino acid or peptide, such that reaction of the amino or substituted amino group of the coupled amino acid or peptide with a carboxyl group or an activated carboxyl group of a further amino acid or peptide is facilitated,

D) in those cases where step (C) has been performed, reacting said amino or substituted amino group of the last-coupled amino acid or peptide with a carboxyl group or an activated carboxyl group of a further *N*-protected amino acid or peptide so as to form a peptide bond between the two moieties,

E) in those cases where step (D) has been performed, optionally removing the *N*-protecting group from an *N*-protected amino or

substituted amino group of the last-coupled *N*-protected amino acid or peptide, such that reaction of the amino or substituted amino group of the latter amino acid or peptide with a carboxyl group or activated carboxyl group of a further *N*-protected amino acid or peptide is facilitated,

F) in those cases where step E) has been performed, repeating steps D) and E) a desired number of times,

G) removing protecting groups possibly remaining on the amino acid moieties of the coupled peptide or protein chain.

44. A method as claimed in claim 43, wherein a peptide having side chains in protected form is coupled in step (B), and steps (C)-(F) are not performed.

45. A method as claimed in claim 43 or 44, wherein a supported, functionalized, polystyrene-grafted polymer substrate as claimed in any of claims 12 to 14 or claim 42 is employed.

46. A peptide- or protein-bearing polystyrene-grafted polymer substrate prepared by a method as claimed in any of claims 43 to 45.

47. A method for the preparation of a polystyrene-grafted polymer substrate bearing a peptide or protein thereon and supported on a substantially chemically inert and optionally light-transparent support material, the method comprising the steps of:

A) providing a supported polymer substrate grafted with polystyrene chains, said polystyrene chains optionally further bearing substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000, at least part of the polystyrene chains of the polystyrene-grafted polymer substrate being functionalized with a chemical functionality facilitating the formation of a substantially permanent covalent anchoring linkage between the polystyrene moiety and a peptide or protein in its native state,

the functionalized, polystyrene-grafted polymer substrate being supported on a substantially chemically inert and optionally light-transparent support material, and

B) coupling said peptide or protein to the functionalized polystyrene moiety.

48. A method as claimed in claim 47, wherein a supported, functionalized, polystyrene-grafted polymer substrate as claimed in any of claims 12 to 14 or claim 42 is employed.

49. A polymer substrate grafted with polystyrene chains and to which a peptide or protein is coupled, said polystyrene-grafted substrate preferably being fashioned in a form selected from the group consisting of a microtiter plate, a test tube, a beaker, a flask, a tray, a Petri dish, a strip, a rod and a fibre, and optionally being supported on a substantially chemically inert and optionally light-transparent support material, said polystyrene chains optionally further bearing substantially non-reactive substituents, the estimated molecular weight of substantially all of the polystyrene chains grafted to the polymer, not including optional substituents, being at least 200,000.

50. The use, in a bioassay procedure for the detection or quantitative determination of a biologically active species, of a functionalized, polystyrene-grafted polymer substrate as claimed in any of claims 1 to 14 or claim 41 as a carrier for the substantially permanent covalent attachment of a peptide or protein having an epitope or binding site that can bind to a complementary binding site or epitope on said biologically active species.

51. The use as claimed in claim 50, said biologically active species being an antibody, and said peptide or protein being an antigen which undergoes an antigen/antibody reaction with said antibody.

52. The use as claimed in claim 50, said biologically active species being an antigen, and said protein being an antibody which undergoes an antigen/antibody reaction with said antigen.

53. The use as claimed in claim 51 or 52, said bioassay being performed using an ELISA technique.

54. The use, in a bioassay procedure for the detection or quantitative determination of a biologically active species, of a peptide- or protein-bearing polystyrene-grafted polymer substrate as claimed in any of claims 15 to 21 or claim 46, said peptide or protein having an epitope that can bind to a complementary binding site on said biologically active species.

55. The use as claimed in claim 54, said biologically active species being an antibody, and said peptide or protein being an antigen which undergoes an antigen/antibody reaction with said antibody.

56. The use as claimed in claim 55, said bioassay being performed using an ELISA technique.

70

Boc-Ala-Asp(OBzl)-Lys(2Cl-Z)-Ala-Asp(OBzl)-

76

Val-Asp(OBzl)-Val-Leu-Thr(Bzl)-Lys(2Cl-Z)-

84

Ala-Lys(2Cl-Z)-Ser(Bzl)-Gln-OCH_2-C_6H_4-CH_2-CO-

NH-CH_2-polystyrene-grafted polyethylene sheet

Fig. 1

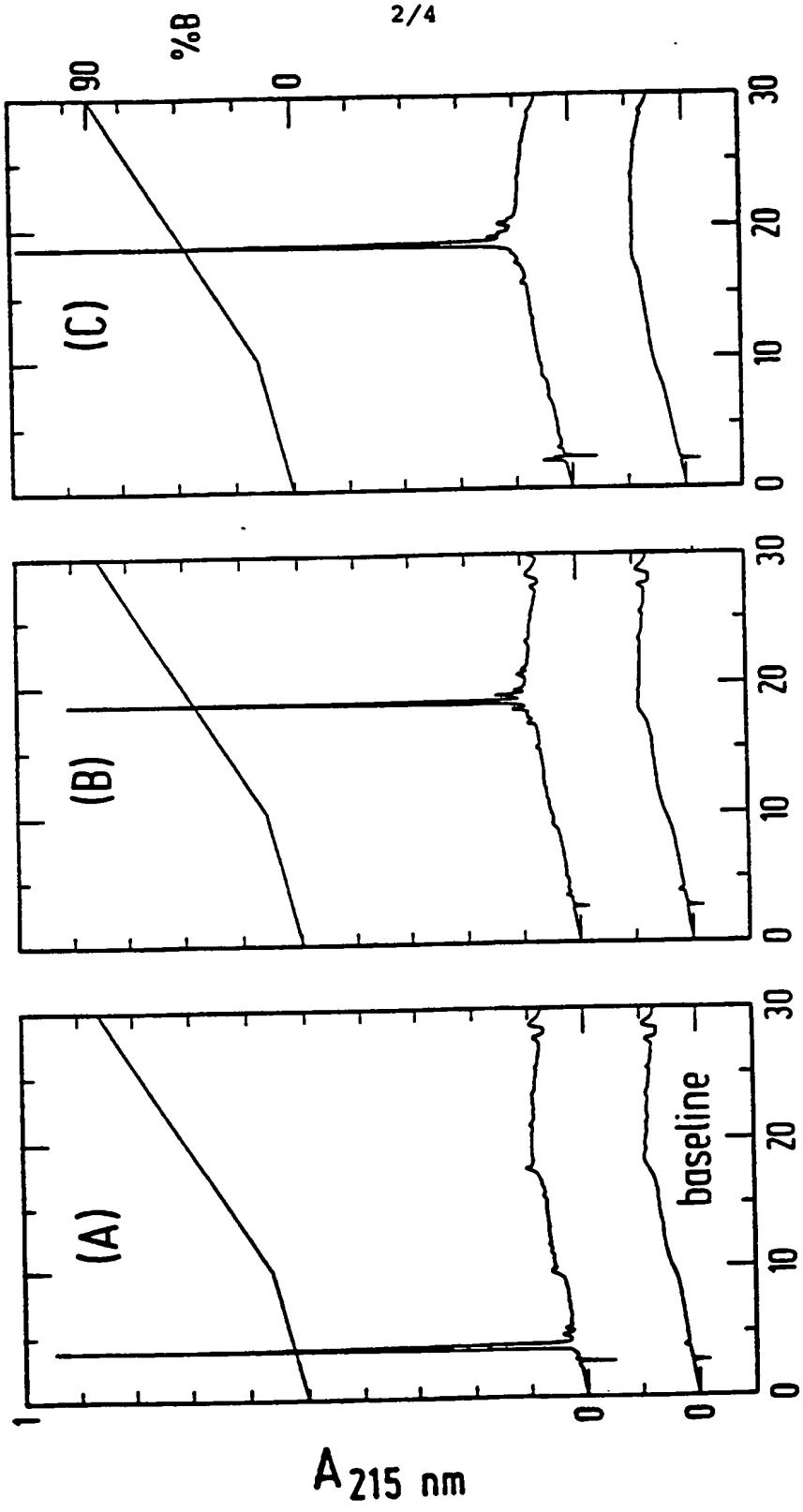
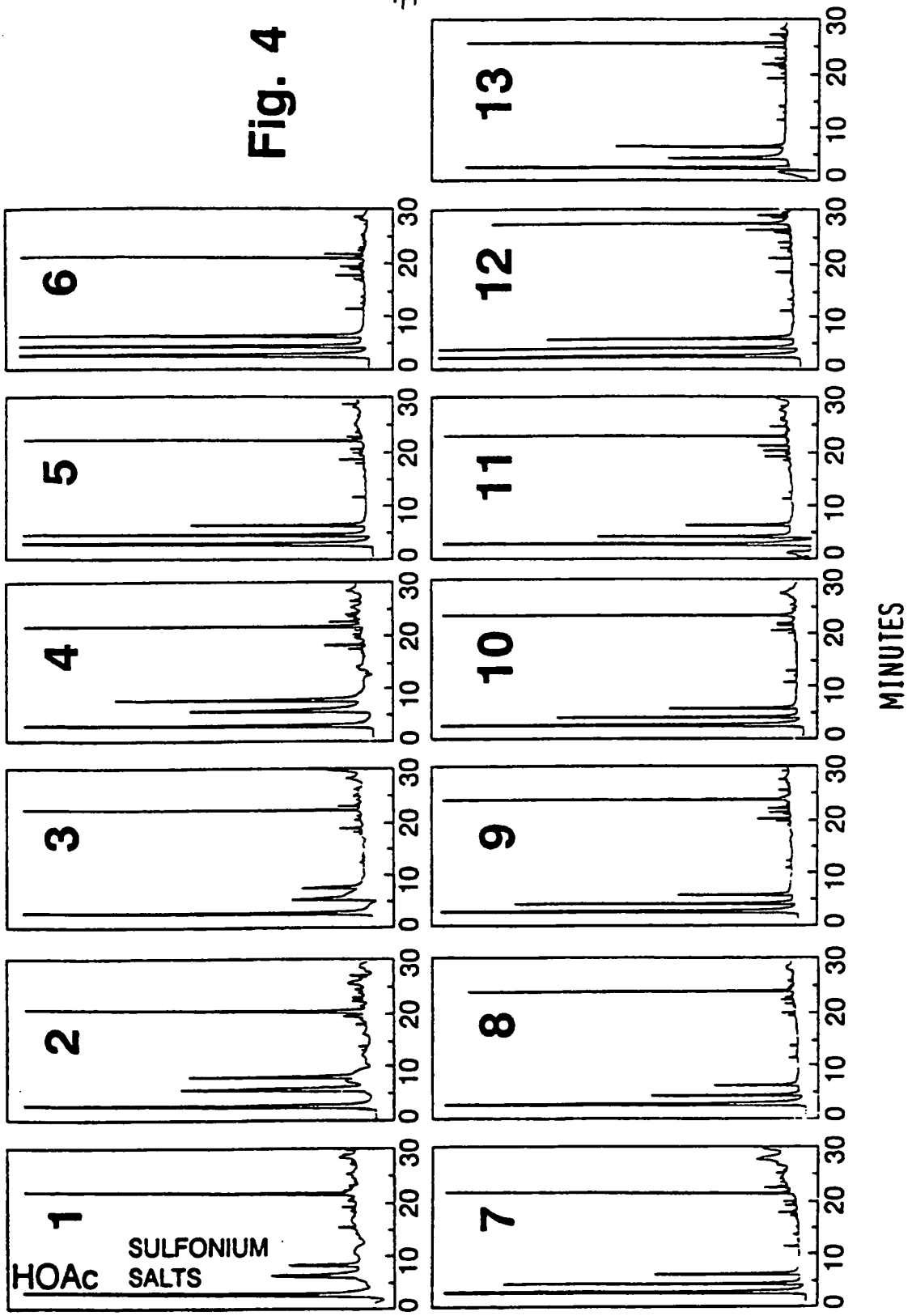


Fig. 2

	PEPTIDE	SEQUENCE
		7 12 14 21
1	MELITTIN-(7-21)	LYS-VAL-LEU-THR-THR-GLY-LEU- PRO-ALA-LEU-ILE-SER-TRP-ILE-LYS-OH
2	[D-PRO^{14}]MELITTIN-(7-21)	LYS-VAL-LEU-THR-THR-GLY-LEU-D-**PRO**-ALA-LEU-ILE-SER-TRP-ILE-LYS-OH
3	[GLY^{14}]MELITTIN-(7-21)	LYS-VAL-LEU-THR-THR-GLY-LEU- **GLY** -ALA-LEU-ILE-SER-TRP-ILE-LYS-OH
4	[LYS^{14}]MELITTIN-(7-21)	LYS-VAL-LEU-THR-THR-GLY-LEU- **LYS** -ALA-LEU-ILE-SER-TRP-ILE-LYS-OH
5	[ASP^{14}]MELITTIN-(7-21)	LYS-VAL-LEU-THR-THR-GLY-LEU- **ASP** -ALA-LEU-ILE-SER-TRP-ILE-LYS-OH
6	[ASN^{14}]MELITTIN-(7-21)	LYS-VAL-LEU-THR-THR-GLY-LEU- **ASN** -ALA-LEU-ILE-SER-TRP-ILE-LYS-OH
7	[SER^{14}]MELITTIN-(7-21)	LYS-VAL-LEU-THR-THR-GLY-LEU- **SER** -ALA-LEU-ILE-SER-TRP-ILE-LYS-OH
8	[LEU^{14}]MELITTIN-(7-21)	LYS-VAL-LEU-THR-THR-GLY-LEU- **LEU** -ALA-LEU-ILE-SER-TRP-ILE-LYS-OH
9	[PHE^{14}]MELITTIN-(7-21)	LYS-VAL-LEU-THR-THR-GLY-LEU- **PHE** -ALA-LEU-ILE-SER-TRP-ILE-LYS-OH
10	[D-PHE^{14}]MELITTIN-(7-21)	LYS-VAL-LEU-THR-THR-GLY-LEU-D-**PHE**-ALA-LEU-ILE-SER-TRP-ILE-LYS-OH
11	[VAL^{14}]MELITTIN-(7-21)	LYS-VAL-LEU-THR-THR-GLY-LEU- **VAL** -ALA-LEU-ILE-SER-TRP-ILE-LYS-OH
12	[LEU^{12}, GLN^{14}]MELITTIN-(7-21)	LYS-VAL-LEU-THR-THR-**LEU**-LEU- **GLN** -ALA-LEU-ILE-SER-TRP-ILE-LYS-OH
13	[LEU^{12}, LYS^{14}]MELITTIN-(7-21)	LYS-VAL-LEU-THR-THR-**LEU**-LEU- **LYS** -ALA-LEU-ILE-SER-TRP-ILE-LYS-OH

Fig. 3

Fig. 4


1
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12
13
HOAc
SULFONIUM SALTS
0 10 20 30
MINUTES
A 215 nm

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00062

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 17/08, C 12 N 11/08, C 07 K 1/04, 3/06, C 08 F 255/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 K; C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	WO, A1, 90/02749 (FORSKNINGSCENTER RISØ) 22 March 1990, see the whole document	1,2,4-9, 11-15, 17-25, 29-36, 38-47, 49
X	US, A, 3981775 (KENYON ET AL.) 21 September 1976, see the whole document	1,2,8,9, 11-13, 22-24, 35,38, 39,41, 42,47
X	GB, A, 1234982 (FARBWERKE HOECHST AKTIENGESELLSCHAFT) 9 June 1971, see the whole document	1,2,8,9, 11-13, 22-24, 35,38, 39,41, 42

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
4th June 1991	1991 -06- 10
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Mikael Bergstrand

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	US, A, 3795664 (G.W. TREGEAR ET AL.) 5 March 1974, see the whole document --	1-56
A	GB, A, 1344706 (F. HOFFMANN-LA ROCHE & CO. AKTIENGESELLSCHAFT) 23 January 1974, see the whole document --	1-56

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00062

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 91-04-30 The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		AU-D-	7722475	76-07-15
		CH-A-	602781	78-08-15
		DE-A-B-C	2501840	75-07-24
		FR-A-B-	2258395	75-08-18
		GB-A-	1466033	77-03-02
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		JP-A-	50105884	75-08-20
		JP-B-	58015119	83-03-24
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		DE-A-	1593880	70-10-29
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		AU-D-	2745467	70-03-05
		GB-A-	1218459	71-01-06
GB-A- 1344706	74-01-23	DE-A-	2109027	71-09-09
		FR-A-	2078974	71-11-05
		NL-A-	7102517	71-08-27